Manipulating gene expression in DRG sensory neurones

Anjan Seereeram

Department of biology

UCL

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Mammalian sensory neurones that respond to tissue damaging stimuli are known as nociceptors. Activation of these neurones can induce a sensation of pain. The aim of this thesis was to develop methods to manipulate gene expression in rodent sensory neurones, in order to understand more about the molecular mechanisms involved in pain pathways.

Three approaches were compared: viral gene delivery, antisense gene knock-down, and transgenic knock-out mice. Firstly, novel recombinant Herpes simplex viruses were used to infect sensory ganglia, and the efficiency of gene transfer assessed using histochemical markers. Secondly, antisense oligonucleotides directed against genes involved in determining neuronal excitability were delivered intrathecally, and the effects on gene expression and behaviour were assessed. Finally, two transgenic mouse lines in which the bacterial recombinase Cre was expressed downstream of sensory neuron specific promoters were analysed. These mice can be used to delete genes flanked by lox-p sites in a tissue-specific manner. The pattern of expression of Cre recombinase was assessed using reporter mice that express beta-galactosidase down stream of lox-p flanked stop (poly-adenylation) sites.

Experiments with modified Herpes simplex viruses expressing green fluorescent protein (GFP) showed that injection of virus into the sciatic nerve resulted in more infected DRG neurones than footpad injection. A maximum transduction level of 7.9 ± 1.3 % of all neurones was achieved one month after sciatic delivery. The majority of transduced neurones (67 ± 1%) were large-diameter neurones. This subpopulation is not generally responsible for the transmission of noxious stimuli. Only 3.4 ± 0.8% of peripherin positive neurones (a population that includes the majority of nociceptors) were successfully transduced. This level of transduction was too low to be used effectively. Vectors expressing VP22 a protein that enhances cell-to-cell viral spread were made, but were unstable. The use of these HSV vectors is thus limited to experimental situations where infection of only a few cells is adequate (for example, examining the function of secreted molecules).

Experiments with labelled antisense probes gave much higher rates of neuronal transduction in comparison to viral transduction methods (>80%). Unlike HSV-vectors however, there was no preferential transduction of neuronal cells, and all cell types were susceptible to oligonucleotide uptake. Intrathecal administration of antisense oligonucleotides directed against the annexin light chain protein p11 resulted in lowered levels of expression of the sodium channel $Na_V1.8$ that requires p11 for insertion into the plasma membrane. Behavioural experiments were carried out to investigate the changes in both normal sensory function, and the alterations in inflammatory-related hyperalgesia after the administration of p11 antisense molecules. It was shown that this approach could successfully modulate the subcellular location of $Na_V1.8$ protein. This was coupled to specific reductions in the hyperalgesic response elicited in rats after peripheral NGF application (an inflammatory agent). The technique of intrathecal antisense administration thus had a more general utility than the use of HSV vectors and may provide a method of down-regulating nociceptive specific proteins leading to reductions in nociception and pain.

The properties of two Cre-expressing transgenic mouse lines were examined. Ncx is a homeobox transcription factor expressed in neural crest-derived tissues. Dorsal root ganglia contain cells which are derived from the neural crest. The subpopulation of neurones in the adult mouse which continue to express genes under the control of the Ncx promoter was therefore investigated to determine its utility. In the adult it was found that a subset of sensory neurones located in the lower DRG (Lumbar/ Cervical) continues to express Ncx in the adult mouse. Positive expression was also located in a number of other tissues in the adult including the Cranial nerve ganglia, sympathetic ganglia and the enteric ganglia. It was found that the majority of enteric ganglia also express Ncx into adulthood. This mouse line can therefore be utilised to target these cells efficiently. The subpopulation of DRG neurones, which continue to express Ncx into adulthood, may represent a sensory neuronal population, which innervates the viscera. This line could therefore find utility in investigating visceral pain pathways.

Finally, a mouse expressing Cre only in nociceptors was used to examine the effect of deleting a neurotrophic factor specifically in this subpopulation. Tissue-specific BDNF mice were found to have dramatic deficits in pain pathways. These include a reduced response to acute thermal noxious stimuli, which involve central signal

processing in the dorsal horn (Hot-plate test), a reduction in the response elicited by low grade acute mechanical stimuli (von-Frey's test at values under 1g only) and a reduction in the thermal hyperalgesia evoked by an inflammatory stimulus (NGF). Also, the centrally mediated second phase of the formalin test was reduced 2.5 fold in the knock-out animals compared to wild-type littermates. These results point to a dramatic and specific role for BDNF in the processing of noxious stimuli.

In summary, it is clear that a variety of methods can be used to manipulate gene expression in sensory neurones to examine mechanisms involved in nociception. In this thesis, successful experiments using antisense oligonucleotides and nociceptor-specific Cre-expressing mice were carried out. It is concluded that the most effective route to manipulating nociceptor gene expression is the use of transgenic mice, but that antisense strategies have some advantages in terms of simplicity and speed. The use of HSV vectors is limited at present due to the low nociceptor transduction efficiency that can be achieved by relatively non-invasive delivery methods necessitating further improvements in vector design before their full potential can be realised.

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Abbreviations

AA - Arachidonic acid
AMPA - α-amino-3-hydroxy-5-methyl-4-isoazoleproprionic acid
BDNF - Brain derived growth factor
BHK - baby hamster kidney cell line
BSA - Bovine serum albumin
CGRP - Calcitonin gene-related peptide
CNS - Central nervous system
DMEM - Dulbecco's modified Eagle medium
DNA - Deoxyribose nucleic acid
DRG - dorsal root ganglia
EDTA - Ethylene diamine tetra-acetic acid
FGM - Full growth medium
Ficoll - multi-branched hydrophilic sucrose polymer
GABA - γ-aminobutyric acid
GDNF - Glial cell-line derived neurotrophic factor
HEPES - N-2-Hydroxyethylpiperazine-N'-2-ethanosulfonic acid
HMBA - Hexamethylene bisacetamide
HSV - herpes simplex virus
IB4 - α-D-galactosyl plant lectin
ICP - infected cell protein
MaM49 - BHK cells expressing ICP27 and ICP4 HSV proteins
mGLUR - metabotropic glutamate receptor
NF200 - Neurofilament 200 (N52)
NGF - Nerve growth factor
NMDA - N-methyl-D-Aspartate
PKCγ - Gamma form of protein kinase C
PNS - Peripheral nervous system
PVP - Polyvinyl pyrrolidone
Rpm - revolutions per minute
RT - room temperature
SC - Spinal cord
SCG - superior cervical ganglia
SDS - Sodium dodecyl sulphate
SFM - Serum free medium
SNS - Sensory neuron specifoc sodium channel
SP - Substance P
ssDNA - single stranded DNA
TrkA - tyrosine kinase receptor A
TrkB - tyrosine kinase receptor B
TrkC - tyrosine kinase receptor C
TTX - Tetrodotoxin
TTXr - Tetrodotoxin resistant
TTXs - Tetrodotoxin sensitive
VGSC - Voltage-gated sodium channel

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Chapter 1: Genetic manipulation of pain pathways.

1.1 Introduction

Anatomical, electrophysiological and pharmacological approaches to the study of pain are well established and continue to provide valuable insights into the functioning of the nervous system. The possibility of genetic manipulation of cells *in vivo* has created new possibilities for the investigation of pain pathways. Through this approach a more precise assessment of gene function is expected to be obtained.

Many genes have now been directly or indirectly linked to nociception (the detection of a painful stimulus) and the generation of pain (Julius and Basbaum, 2001). Although conventional approaches such as electrophysiology can provide strong evidence for a genes role in nociception and pain, behavioural experiments are imperative to establish conclusively the role of a gene in pain pathways (Bon et al., 2002).

Many genes which are implicated in pain pathways also serve other roles (Cockayne et al., 2000; McMahon and Bennett, 1999; Waxman et al., 2002). The ablation of these gene products can therefore lead to disruptions in multiple pathways complicating the specific assessment of a gene's role in nociception and pain. The use of tissue specific, inducible knock-outs is one method of circumventing this problem.

In order to examine the role of a gene in pain pathways it is necessary manipulate its sequences both *in vivo* and *in vitro. In vitro* experiments are quick, efficient and can be used to rapidly determine the importance of a particular sequence in cellular functions. The characterization of the behavioural consequences, of the manipulation of this gene however, can only be studied *in vivo.* Although certain lines of evidence from *in vitro* experiments, such as electrophysiological data, can provide clues which can help to predict the role of a gene, *in vivo* studies allow its contribution in nociceptive pathways to be assessed more completely, and within the context of the fully functional system. Thus, *in vivo* experiments are invaluable in the validation of a gene of interest as a potential target for pharmacological intervention with a view to alleviating or elimination of pain in humans.

To characterise molecular mechanisms controlling an organism's response to pain within the peripheral nervous system, efficient methods of gene targeting of nociceptors

are required. Nociceptors form a subset of sensory neurones whose somata are collected together into dorsal root ganglia (DRG) and comprise the first order neurones of the afferent pathway (Besson, 1999) and are specialised damage sensors. These cells provide an important target for genetic manipulation aimed at modulating pain. The investigation of the molecular basis of pain detection and transmission by these cells requires techniques which can differentiate nociceptive neurones from other sensory neurones present in the DRG. The ability of a protocol to achieve this discrimination is central to the successful elucidation of the role of a gene in nociception. This thesis explores a variety of approaches which may provide the means of specifically targeting defined subsets of sensory neurones in the peripheral nervous system.

Several genetic manipulation techniques that are in current usage allow the manipulation of genes of nociceptive cells (in addition to other non-nociceptive cell populations) *in vivo*, including antisense-based protocols, RNA interference strategies, viral-vector based gene delivery as well as transgenic animal engineering. The degree to which each of these approaches can discriminate and selectively influence gene expression in nociceptors varies. This thesis illustrates several attempts to address this shortcoming and achieve gene manipulation specifically in nociceptors or other defined subsets of neurones.

Ultimately, the establishment of transgenic animals where a gene of interest is either over-expressed or ablated are both necessary to define the functions of the gene product within normal and painful states (Joyner, 1994). Furthermore, the use of animals in which a gene of interest has been manipulated only in defined subsets of cell types will lead to more informative data (Orban et al., 1992). The development of transgenic animals with nociceptor specific gene manipulation will greatly aid in the elucidation of pain pathways. To this end two Cre-expressing transgenic lines were investigated to determine their potential usefulness at achieving nociceptor-limited gene manipulation.

These experiments form part of a wider study aimed at the development of a range of complementary transgenic animals which exhibit defined and conditional expression of Cre-recombinase. A concert of these Cre-expressing strains (in complementary subsets of sensory neurones for example) can then be used to ablate a gene of interest in these subpopulations and further define its role. These complementary crosses can also be

subjected to identical nociceptive testing in order to determine the site of production/activity of the ablated gene (Tsien et al., 1996; Wagner et al., 2001).

The refinement of animal models which mimic or reflect various pain states experienced by humans has been developed (Crawley and Paylor, 1997). These paradigms allow the role of a particular gene to be assessed in various pain pathways. These models also allow the extrapolation of behavioural changes observed in animals to human clinical pain states.

This aim of this study is twofold, firstly to make a comparison between the use of viral vectors, transgenic knock-out animals and antisense technology to achieve effective genetic manipulation in the peripheral nervous system and secondly to utilise the best approach to target specifically genes involved in nociceptive pathways in defined populations of sensory neurones (in DRG) in an attempt to affect pain related behaviours *in vivo.*

1.2 *in vivo* genetic manipulation techniques

1.2.1 Transgenic studies

The development of transgenic techniques in the 1970's allowed the modification of animal genomes to be achieved in a controlled manner. The first experiments inserted the transgenic sequence randomly into the embryonic genome of the target species (Palmiter and Brinster, 1985) through the use of pronuclear injection techniques. The main drawback of these first-generation transgenics centred on the lack of control over the insertion site of the transgene, and hence the possibility of the recombination event either bringing the construct into an area of the genome where it is disabled though the presence of endogenous repressor sequences nearby, or where it disrupted the proper functioning of another gene leading to non-viability or confounding effects. In either case the interpretation of the result becomes difficult.

An improved protocol, exploiting homologous recombination to direct the transgene sequence precisely was developed to correct the problems associated with random integration techniques (Capecchi, 1989). This methodology allows the replacement of the targeted endogenous sequence with a transgenic one. The transgene may be engineered to

include frame-shift mutations or abolish whole exons to ablate the gene, or utilize more subtle changes to introduce stop codons or alter the amino-acid constitution of the resultant protein (Mogil and McCarson, 2000). It is imperative to assess these transgenics in a wide variety of behavioural assays in order to determine if the genetic intervention has had any overt effects which can compromise the interpretation of results (Dahme et al., 1997).

Many studies have successfully used targeted transgenic manipulation to investigate the function of nociceptive genes in pain pathways. The functions of diverse genes in nociceptive pathways, including the genes which encode the voltage-gated sodium channel $Na_v1.8$ (Akopian et al., 1999); NGFb (Nerve Growth Factor β-subunit)(Crowley et al., 1994); and Tac1 (preprotachykinin1 PPT-A) (Cao et al., 1998), have been clarified through the development of transgenic animals either over-expressing or ablating these genes. The changes in nociceptive behavioural patterns, as measured by standard behavioural screening protocols (Crawley and Paylor, 1997), after gene manipulation, have been both augmented as well as reduced (Mogil and McCarson, 2000).

It is important to assess the positive attributes as well as the drawbacks of using transgenic approaches particularly when applied to the study of nociception and pain pathways. Firstly, the deletion of a gene may lead to major and lethal developmental deficits in the nervous system (Joyner, 1994) and/or other systems precluding the use of this technique in the investigation of its role in nociception and pain pathways. Several examples of such genes have come to light; Kinesin member 1A coded by KIF1A (Yonekwa et al., 1998)and L1 cell adhesion molecule (L1cam) (Dahme et al., 1997) are two examples where transgenic ablation has lead to aberration in the development of the nervous system and therefore precluded the use of this system in a proper assessment of these genes in the mature nervous system.

The application of transgenic manipulation techniques allows the study of proteins where they exist in several transcriptional forms (Wynick et al., 1998), exist as several subtypes or as multimeric proteins (composed of multiple, distinctly-encoded subunits) (Minami et al., 1997) to be achieved. Each of these situations present important barriers to their study using conventional techniques such as the requirement of discriminatory pharmacological agents in order to study receptor subtypes or compensatory effects of

alternative splice variants. In these cases, the use of transgenic technology has allowed the collection of data much more easily that would otherwise have been possible.

At present transgenic technology is only practical for the manipulation of *Mus musculus,* requires a relatively large expenditure of both time and resources and may be compromised by compensatory effects from the unaltered allele. The completion of the sequencing of the human, rat, mouse genomes amongst others has created a large resource which can be applied to nociceptive gene identification. However the disadvantages listed above preclude the use of transgenic techniques to screen the pain-related functions of such genes. There have been some attempts to use blind random chemical mutagenesis followed by phenotypic screening to elucidate novel genes, but this has not been applied to pain research specifically and is again limited by cost, time constraints and the other disadvantages listed above.

The use of the Cre-loxP system (Sauer and Henderson, 1988) has been combined with targeted transgenic knock-out techniques to achieve a greater degree of control of both the temporal induction and tissue specificity of the gene ablation event (Tsien et al., 1996; Gu et al., 1993). The introduction of this approach with its associated temporal and spatial control has allowed the generation of transgenic animals which circumvent or entirely overcome many of the problems encountered using normal total transgenic animals.

1.2.1.1 *CRE-loxP DNA-excision system*

The bacteriophage P1 enzyme Cre recombinase can be employed to both delete and express genes in cell lines or animals (Sauer and Henderson, 1988). The Cre enzyme recognises a 34bp target sequence called the loxP site (Hamilton and Abremski, 1984). The enzyme is able to effect a recombination event between two cis located loxP sites leading to the loss of intervening sequence or the inversion of the spacer region or the insertion of a DNA stretch if the two are located in a trans arrangement to each other.

The Cre-loxP system is self contained and does not require any additional co-factors and has been shown to be effective in eukaryotic systems (Sauer and Henderson, 1988) including mice (Orban et al., 1992). Importantly the random occurrence of an exact 34bp loxP site in the entire eukaryotic genome is highly unlikely making the control of the recombination event predictable (Nagy, 2000) although some studies have noted the possibility of similar eukaryotic sequences which can rarely be recognised by the Cre-recombinase enzyme and mediate activity (Thyagarajan et al., 2000).

The excision of stretches over 400bp has been catalysed through the use of this system using a strong promoter to drive the Cre expression (Niwa et al., 1991). Here excision efficiencies around 50% were achieved.

A key step in the creation of an effective strategy is the identification of a suitable promoter to drive the Cre expression. This selection allows the tailoring of the excision event temporally and/or spatially allowing greater control over the manipulation of the genome. These concepts have come to play an important part in the efficient utilization of the Cre-recombinase system in pain studies.

The addition of an additional level of control by linkage to inducible systems utilizing tetracycline/ doxycyline (Gossen and Bujard, 1992) or oestrogen/ tamoxifen (Berry et al., 1990) activated sequences has further enhanced the Cre-excision system and allow a greater level of temporal control to be exerted on the excision events. This approach may provide the key to overcoming genetic manipulation of essential developmental genes which cannot be ablated at an early stage without serious consequences.

Several inducible Cre enzymes (St Onge et al., 1996; Metzger et al., 1992; Feil et al., 1996; Danielian et al., 1998), have been generated in recent years which rely upon the

introduction of an exogenous ligand (tamoxifen/ tetracycline) to either activate or terminate Cre-expression in an inducible manner, controlling the timing of the excision events (Lewandoski, 2001; Metzger and Chambon, 2001; Leone et al., 2003; Lindeberg et al., 2002).

The discovery of nociceptive specific regulatory sequences which can be expoited to examine the role of genes in pain pathways is another important line of investigation. The regulatory sequences of a sodium channel ($Na_V1.8$) expressed exclusively in nociceptors is one such sequence. In this thesis, work is presented which exploits a $Na_V1.8$-limited Cre-expressing mouse line (Stirling et al., 2003) to examine the role of a neurotropic factor important in inflammatory pain.

The crossing of this nociceptor-specific Cre-expressing strain with a second strain which contains a floxed neurotrophic factor BDNF (allowing recognition by the enzyme and subsequent mediation of excision of the BDNF sequence preventing its transcription) (Rios et al., 2001) has been performed to specifically eliminate the production of BDNF in nociceptive cells of the peripheral nervous system. The role of nociceptor-derived BDNF in pain pathways can therefore be investigated.

These experiments should validate BDNF as a target for pharmacological agents used to treat pain. BDNF has been previously implicated in the transmission of nociceptive information at the central terminals of nociceptive neurones in the dorsal horn (Pezet et al., 2002c), however the source and relative contribution of this BDNF could not be adequately assessed due to limitations in the techniques used in these studies. The work presented in this thesis validates and extends these earlier findings.

1.2.2 Viral vectors – use in over-expression and gene ablation studies

Manipulation of the eukaryotic genome of model organisms can also be accomplished through the use of modified viral vectors. Viruses are small infectious particles adapted to infect particular cell types and "hijack" cellular machinery to produce foreign viral genes leading eventually to the production of new virions.

Many of the mechanisms which have evolved in viruses are therefore directly applicable to the task of eukaryotic genome modification. Several different classes of viruses have provided the raw material, from which vector systems to deliver exogenous

sequences, have been developed. These include replication competent viruses which encode their genetic information in DNA (herpesviruses [HSV], adenoviruses) as well as RNA (lentiviruses, retroviruses) or replication-incompetent degenerate viruses which require helper-virus co-infection in nature to provide essential functions (adeno-associated virus [AAV]).

Several important considerations are common to the development of any vector from a wild-type virus.

1. The safety profile of the vector.
2. The toxicity/inertness of the vector in the target cells.
3. The infection profile (specifically cell types and timing of expression).
4. The size of transgene which can be accommodated.
5. Ease of production of the vector.
6. Efficiency of direct infection of tissue versus indirect infection using explanted cells taken from the target organism.
7. Modifications of the vector to include inducible elements to effect further control over transgene functions.

These items will be described for four viral vectors systems currently being investigated with particular emphasis placed on their potential utility in the peripheral nervous system (PNS)

1.2.2.1 Herpes simplex virus-based vector systems

The HSV type 1 virus has many characteristics which have allowed it to be successfully used to develop neuronal vector systems (Burton et al., 2002; Lilley et al., 2001a; Goins et al., 1997).

HSV-1 based vectors exhibit good toxicity profiles whilst maintaining a wide infection range. As a large double-stranded DNA virus (152kb) with large stretches of non-essential genes, HSV-1 derived vectors have the ability to incorporate large transgenes and have natural immune-avoidance mechanisms resulting in the establishment of long term infection and gene expression.

The main advantages of HSV-based viral vectors over other viral vectors centre upon its ability to target neurones effectively with low toxicity (Krisky et al., 1998), including its ability to track retrogradely up nerves from peripheral infection sites to cell bodies using a dynein-mediated mechanism (Ye et al., 2000).

The establishment of a latent-infection separate from a lytic one in which virus production is occurring actively is another advantage. In the latent stage of the life cycle only two small RNA transcripts (termed LAT's) have been identified in a wild-type infection. The reduction in viral proteins results in a lower cytotoxicity and allows the virus to exist for a long time undetected in the cell (Burton et al., 2002). By exploiting the sequences (promoter, enhancer regions) responsible for the direction of these transcripts during latency vectors which also allow the long-term expression of transgenes have been engineered (Lachmann and Efstathiou, 1997). The development and use of HSV-1 based vectors is expanded further in chapter 3 along with their use in targeting neurones of the peripheral nervous system.

1.2.2.2 Adenoviruses and Adeno-associated viruses; Vector development and usage.

1.2.2.2.1 Adenovirus-based vectors

Adenoviruses are large double-stranded DNA viruses which are currently being used to effect gene transfer *in vitro*, and *in vivo.* Adenovirus-based protocols have also shown promise in DNA vaccine applications. Adenoviruses can transduce a wide variety of mitotic and post-mitotic cell types including neurones, with the viral genome remaining episomal avoiding deleterious effects in the host cell due to insertional mutagenesis (Horowitz, 1990).

Vectors have been derived with deletions in essential genes rendering them safe. These deletions have also allowed the incorporation of transgenic sequences up to 8kb, depending on the extent of viral gene deletions. These replication-deficient vectors have also been grown to high titres relatively easily (He et al., 1998; Gorziglia et al., 1996).

The safety of adenoviruses has been well demonstrated as they have been extensively used as live vaccines without any associated tumourogenesis in millions of patients (Strauss, 1984). Unfortunately, they do not evade host immune systems

effectively and are rapidly cleared limiting the duration of transgene expression. The ability to re-infect subsequently is also negligible due to host immune responses to the adenoviral challenge.

Three approaches have generally been used to create recombinant adenoviruses containing a transgenic sequence. The first involves the direct cloning of the transgene into the adenoviral genome. This method has been limited by the dearth of unique restriction enzyme sites in the adenoviral genome (Rosenfeld et al., 1991).

The second method exploits homologous recombination events in packaging cell lines engineered to express the proteins deleted from replication defective viral lines *in trans*. The manipulation of the shuttle vectors containing small flanking regions of the adenoviral genome is done in bacterial cells making cloning easy and selection of recombinants simple (Mittal et al., 1993; Becker et al., 1994).

A third method has moved the recombination event from eukaryotic cells to prokaryotic cells where homologous recombination occurs at a much higher frequency. This was achieved by engineering the backbone sequence into another bacterial strain in a super-coiled form. The cloning is performed with conventional techniques. The manipulation in prokaryotes also allows more rapid screening protocols to be used and therefore a reduction in the number of ligation events required to produce the recombinant vector (He et al., 1998).

At present very few adenoviral vector systems have been used to target neurones in the PNS (Somia and Verma, 2000). The main drawbacks to the use of these vectors to transduce neurones in the PNS include; difficulty in achieving high infection rates through non-invasive introduction routes (Glatzel et al., 2000; Huard et al., 1995), dearth of regulatory sequences to drive long-term high-level expression in the target tissue (Glover et al., 2002; Navarro et al., 1999; Xu et al., 2003c) and relative ease of clearance by host immune responses. These caveats have all retarded adenoviral-based intervention protocols (Thomas et al., 2001; Yang et al., 1994). Further investigations to improve these vectors are underway and may lead to the characterisation of adenoviral-vector backbones which exhibit a better cytotoxicity and neuron infective profile which will aid their use in peripheral neuron transduction protocols.

1.2.2.2.2 Adeno-associated virus (AAV)-based vectors

Adeno-associated virus is a small non-pathogenic single-stranded human DNA parvovirus. It is non-enveloped and non-cytotoxic (Samulski et al., 1999). Due to the limited viral genes encoded by its genome it requires the co-infection of replication-competent helper virus (an adenovirus or herpes virus) to provide essential functions leading to a productive infective cycle. When the helper virus is absent the virus reverts to a latent cycle rather that a lytic one (Carter, 1992). AAV manufacture without the help of other viruses has been noted after treatment of infected cells with ultraviolet radiation, gamma irradiation or hydroxyureas (Schlehofer et al., 1986).

Multiple serotypes of AAV have been identified as contaminants in adenoviral cultures but none have ever been associated with human disease and several serotypes have been negatively correlated with the risk of virally-induced cancers (Cukor et al., 1975; de la Maza and Carter, 1981).

AAV in the latent phase can exist integrated into the human chromosome 19 (Weitzman et al., 1994). The rate of latency and hence insertion is very low however and therefore insertional mutagenesis events are rare, an advantage to using AAV-derived vectors to engineer transgenes into host cells. The presence of an episomal location of the AAV genome during latency has also been recorded. In both types of latency (episomal or integrative), no gross morphological or metabolic changes have been noted in cells infected latently with AAV, this remains a major advantage of gene delivery systems based on these viruses (Cheung et al., 1980).

Presently most AAV-derived vectors have been derived from AAV subtypes 2, 4 and 5 which all exhibit differences in their host-cell specificity (Davidson et al., 2000). Recombinant AAV vectors (rAAV) derived from subtype 2, share many important properties of the parent strain including the ability to infect both mitotic and post-mitotic cells such as neurones, and the establishment of latency for the life of the infected host cell in the absence of trigger signals (Wu et al., 2000).

Manipulation of the AAV genome is made simple due to its small size and general absence of viral sequence. The entire genome has been sequenced and shuttle vectors, permissive cells lines and safe helper viruses have all been created which allow the efficient production of recombinant viruses (Haberman et al., 1999; Ferrari et al., 1997).

rAAV vectors have been successfully used to infect and mediate long-term transgene expression in a wide variety of tissues including brain, PNS (neurones but not glia), liver, muscle, retina and vascular epithelia (Samulski et al., 1999). In the peripheral nervous system, AAV-based vectors have delivered a wide range of neurotrophic substances both *in vitro* and *in vivo*, including preprotachykinin-A (Quinn et al., 2000), neurotrophins BDNF (Eaton et al., 2002), NT-3 (Blits et al., 2003) and GDNF (Lu et al., 2003), as well as the μ-opioid receptor (Xu et al., 2003b).

Some of these investigations have produced results which illuminate nociceptive-specific questions occurring within the dorsal root ganglia, the main tissue of interest in the studies presented in this thesis and achieved good transgene expression up to three month post-infection. They all required the invasive technique of direct DRG injection to achieve these results however, which remains the main disadvantage to rAAV-mediated gene delivery to the DRG.

1.2.2.3 Lentiviruses

The lentivirus family has provided several transgene-delivery vector systems including those based on the human immunodeficiency virus-1 (HIV-1) (Mochizuki et al., 1998), feline immunodeficiency virus (FIV) (Kyrkanides et al., 2003) and equine infectious anaemia virus (EIAV) (Wong et al., 2004). Lentivirus-based vectors share the ability to infect post-mitotic cells such as neurones (Naldini et al., 1996) with HSV, Adeno- and AAV-derived vectors (Brun et al., 2003). Vector systems based on HIV-1 and EIAV are the most advanced and show promise in transduction of neuronal cells.

The tropism of lentiviral vectors has been greatly expanded through the process of pseudotyping. Viral tropism is determined by specific interactions between viral surface glycoproteins and surface receptor targets on the membrane of the host cell. The modification and/or replacement of the original envelope (pseudotyping) can alter the infective profile of the vector. (Wong et al., 2004)

The selective infection of neurones both in the CNS and PNS has been greatly enhanced by pseudotyping lentiviral vectors with the envelope derived from vesicular stomatitis virus G-protein (VSV-G) (Anderson et al., 2000; Falk et al., 2002).

The integrative property of lentiviruses also allows the long-term expression of transgenes to be achieved. Unfortunately the possibility of insertional mutagenesis is increased along with this property. Multiply deleted versions of lentiviral vectors together with isolation and characterization of neuron specific promoter/enhancer sequences will increase the usefulness of these vectors. At present there are few reports of efficient transduction of PNS neurones by HIV-1 based lentiviral vector system either *in vitro* or *in vivo* (Ginn et al., 2003; Fleming et al., 2001).

1.2.3 Antisense technology – post transcriptional control

Antisense-mediated has been widely used to downregulate genes in nociceptive pathways. G-protein coupled receptors (Young et al., 1998; Fundytus et al., 2001), voltage- (Lai et al., 2002; Gold et al., 2003; Yoshimura et al., 2001b) and ligand-gated ion channels (Barclay et al., 2002), neuropeptides (Zhu et al., 1997) and second messenger molecules (Gillardon et al., 1997) have all been successfully downregulated using antisense oligonucleotides (AS). Functional outcomes including altered protein levels and behavioural modification have all been achieved through the use of these molecules.

Antisense oligonucleotides are small synthetic chains of oligo-deoxynucleotides designed to interact specifically with mRNA sequences coding for the protein of interest. The interaction of the AS molecule with its target RNA species leads to the down-regulation of the encoded protein. This occurs via two distinct mechanisms, firstly the binding of the AS molecule to the RNA can lead to the degradation of the mRNA by cellular RNases (e.g. RNase H) which recognise the DNA-RNA binding of the AS and mRNA and degrade the target RNA leaving the original AS molecule free to interact with another molecule of mRNA . Secondly, the binding of the AS molecule to the mRNA species can lead to a direct steric impediment in the cell translation machinery, specifically RNA processing steps such as 5'-capping of the primary transcript, inhibition of 3'polyadenylation, splicing interference, steric ribosomal effects as well as translational arrest (Crooke, 2001).

The delivery of AS molecules to the DRG *in vivo* requires invasive surgery. Most commonly the oligomers are injected or fed by catheter into the spinal fluid from where they travel caudally along the spinal column (Lai et al., 2002) and also into the dura

covering the DRG (Honore et al., 2002). The small AS oligos are subsequently found in the cell-bodies of sensory neurones as well as in the spinal cord (Stone and Vulchanova, 2003).

As a consequence of the invasive *in vivo* introduction method required by antisense protocols, confounding inflammatory or metabolic changes such as rises in body temperature may occur. These changes will have effects on the physiological processes under investigation requiring adequate control protocols in order to correctly interpret the data. In practise, commonly used controls use a combination of sham operated animals, administration of vehicle only, mismatched oligonucleotide controls as well as the proposed antisense molecule (Stone and Vulchanova, 2003).

Antisense approaches to the study of nociception and pain can investigate gene functions through ablation studies. It is not possible to achieve over-expression of a gene with this approach however (Crooke, 2001). This remains a disadvantage to these studies as both over-expression and elimination of a gene product is important for the characterisation of its function. Secondly, the efficiency of knock-down achieved though the use of antisense protocols can also be limited if the target mRNA species is well transcribed requiring larger doses of oligos (which in themselves can be toxic). These studies also are limited by the difficulty in targeting the mRNA degradation to specific cell types. Advantageously, it is possible to create oligonucleotides which can bind to a series of mRNA splice variants, allowing for successful regulation of a family of related transcripts which is difficult to achieve with other methods e.g. pharmacological agents.

This thesis describes experiments which exploit antisense technologies to downregulate a sodium channel accessory-protein p11 in order to determine any effects on pain behaviours during inflammation.

The discovery of an accessory protein p11 which could efficiently aid the expression of a sodium current ($Na_V1.8$- related current) *in vitro* (Okuse et al., 2002), a current which had previously been difficult to observe in heterologous expression systems (Akopian et al., 1999), and is directly implicated in transmission of painful stimuli particularly in inflammatory conditions (Kerr et al., 2001), demonstrated that this protein could be a important site for future investigations in controlling the pain associated with inflammation. There are a present no known pharmacological blockers of the p11-$Na_V1.8$

pathway requiring the use of alternative protocols to study the importance off p11 in inflammatory pain.

In order to validate the relative contribution of p11 *in vivo,* work is described which utilized an antisense approach to try and down-regulate the expression of p11 protein and consequently reduce the amount of functional $Na_V1.8$ protein. An antisense sequence directed against the $Na_V1.8$ accessory protein p11, was previously validated for use *in vivo*, with a series of *in vitro* experiments which demonstrated its efficiency at reducing the electrophysiological current associated with the $Na_V1.8$ channel in nociceptors obtained from dissociated DRG cultures (these antisense molecules were directly injected into the DRG neurones) (Okuse et al., 2002). Behavioural consequences of this intervention were therefore sought to determine if specific deficits in inflammatory related pain levels could be achieved therefore lending support to the earlier data.

The delivery of the anti-p11 oligonucleotides directly into the cerebrospinal fluid *in vivo* was investigated to determine if this method would achieve significant delivery to the target tissue leading to behaviourally measurable effects on inflammatory pain conditions which are known to involve $Na_V1.8$ currents. This work should contribute to the validity of pharmacological intereventions in the p11-$Na_V1.8$ interaction as a potential method of allieviating inflammatory pain.

1.3 Molecular mechanisms of pain

1.3.1 Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (www.iasp-pain.org). It can be classified into three major subcategories based upon its context; acute, inflammatory or neuropathic pain. Cancer pain which is sometimes classed separately is most likely an amalgamation of various aspects of neuropathic and inflammatory pain conditions.

The nervous system exhibits a high degree of plasticity in the encoding and manipulation of noxious stimuli received during each of these different states (Corderre et al., 1993; Suzuki and Dickenson, 2000; Dib-Hajj et al., 1999). This can be inferred by the varying levels of pain attributed to an identical stimulus under these differing conditions e.g. allodynia associated with inflammation.

Maintenance of these pain-encoding mechanisms is central to the proper functioning of the system and aberrations lead to abnormal signal generation/transduction and the generation of chronic pain (Zimmermann, 1985). Symptoms and activity arising from normal tissues when challenged with noxious stimuli reflect the intensity, localisation and timing of the initiating stimuli. In contrast, in neuropathic or inflammatory conditions, the generation of the pain from normally innocuous stimuli can generate a painful response (Zimmermann, 2001; Kidd and Urban, 2001). When this occurs as a result of normally innocuous mechanical stimuli it is termed allodynia, this can be contrasted to hyperalgesia which is characterised as an exaggerated response following identical noxious stimuli under differing states.

1.3.2 Nociception

Nociceptors are specialized sensory neurones that are activated by noxious insult to peripheral tissues. Cutaneous and deep somatic tissues are innervated with primary afferent neurones which have three functions in the nociceptive pathway; a) detection of the initial noxious stimuli, b) relay of the resulting message electrically from the primary

sensory neuron to the spinal cord, c) synaptic transfer of this information to second order neurones in the dorsal horn (Mendell et al., 1999; Raja et al., 1999).

Noxious stimuli can be elicited by at least four distinct mechanisms; intense heat and cold, intense pressure, irritant chemicals and tissue damage (Snider and McMahon, 1998). The importance of the detection of these stimuli is self-evident and they serve to warn of impending or ongoing damage. These acute stimuli are therefore important in alerting the organism that there is a dangerous situation. When children born with congenital insensitivity to pain injure themselves, the absence of sensory input during insult can result in severe and permanent damage to tissue.

The central role played by nociceptors in the process of noxious stimuli detection requires that the primary damage-sensing neuron endings be widely distributed all over the body, innervating skin, muscle, joints, and the viscera. The cell bodies of these sensory neurones are located outside the spinal cord and collected together to form ganglia. The vast majority of these ganglia are arranged dorsally just outside the length of the spinal cord and are termed the dorsal root ganglia (DRG) (Willis and Westlund, 1997). The neuron bodies of the majority of nociceptors innervating the head are collected together in the trigeminal ganglia which form a subclass of sensory ganglia distinct from the DRG.

1.3.3 Classification of ganglia neurones

Primary sensory neurones vary substantially in size, peptide content and electrophysiological properties. These neurones have fibres that innervate periphery and viscera where they collect sensory information including noxious stimuli.

Primary sensory neuron cell bodies can be classified according to size into large diameter (>35μm) medium diameter (~25μm) and small diameter (<25μm) classes. These divisions can also be correlated to the degree of myelination present in their axons as well as differences in the intracellular organelles. Various molecular markers have been used to characterise these populations of neurones but as yet the exact relationship between cell size, and function remains inconclusive due to the overlap or sub-group labelling of many markers (Lawson, 1992).

The presence of a 200kDa Neurofilament presence has been used to characterise large diameter neurones, there are at present a few antibodies raised against epitopes

present in this protein with monoclonal N52 and RT97 (raised against a phosphorylated epitope) being the most commonly used (Gotow and Tanaka, 1994; Lawson et al., 1984; Gotow and Tanaka, 1984). Both of these antibodies have been shown to label exclusively myelin-containing neurones (medium to large size). In contrast the small diameter population of neurones can be identified by the presence of peripherin (type III neuron-specific intermediate filament) (Ferri et al., 1990; Zhou et al., 2002).

1.3.4 Classification of peripheral axons

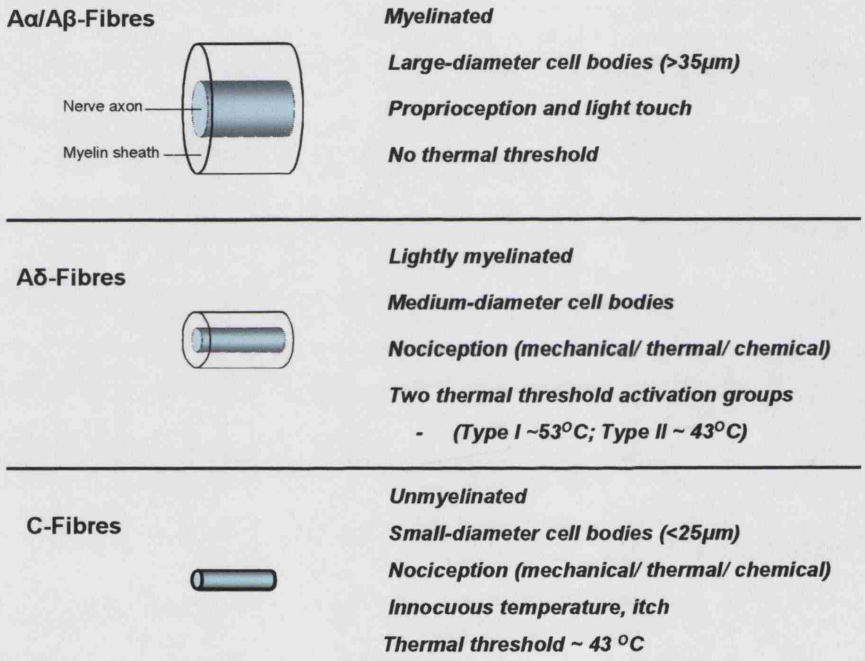


Figure 1. 1 Primary afferent axon subpopulations

Figure1.1: Peripheral nerves have axons which fall into three major categories, a) Large diameter Aα/β myelinated neurones with fast conduction velocities, b) Medium diameter, lightly myelinated Aδ-neurones with slower conduction velocities and c) small diameter, unmyelinated C-fibres.

There are three morphologically different classes groups of axon fibres which can be distinguished, the Aα/Aβ, Aδ and C-fibres. These fibres can be differentiated based upon their relative sizes as well as the presence and quantity of myelination surrounding them.

They also have varying electrophysiological and functional characteristics. Broad relationships can be drawn between axonal properties and cell-body size.

1.3.5 Classification of nociceptors

Nociceptors form a subpopulation of primary sensory neurones and can be largely divided into two main groups based on the axons they bear (Raja et al., 1999). Aδ-fibre nociceptors have thinly myelinated axons, conduct action potentials rapidly (5-30m/s) and have medium- to large diameter cell bodies (~25μm). These neurones are responsible for the mediation of the fast, pricking quality of pain. The second category of nociceptors, the C-fibre neurones have unmyelinated axons, conduct action potentials slowly (generally at velocities of less than 1.0m/s), and have small-diameter (<25μm) cell bodies. The C-fibres comprise approximately 70% of all nociceptors and are responsible for mediating the slow burning component of pain (Stucky et al., 2001).

There appears to also be a small proportion of fibres arising from the Aα/β subgroup of sensory neurones which participate in the transduction of nociceptive stimuli (Lawson, 2002). However, the majority of Aα/β fibres are important in detecting innocuous stimuli and arise from cell bodies with the largest diameter (>30μm). The axons arising from this subpopulation of neurones are well myelinated allowing for rapid action potential conduction velocities to be achieved. They are generally involved in mechanoreception and proprioception (light touch)(Lawson, 2002).

Aδ nociceptors can be divided into two subgroups based on their differential responses to intense heat and tissue damage (inflammatory stimulation), but both classes respond to intense mechanical stimuli (Raja et al., 1999).

Most C-fibre nociceptors are polymodal, responding to noxious thermal and mechanical stimuli, with a small proportion being mechanically insensitive whilst retaining their ability to respond to noxious heat. Additionally most C-fibres are responsive to noxious chemical stimuli such as acid or capsaicin. A small proportion of C-fibres seem to be activated only upon tissue injury and are termed silent or sleeping nociceptors (Schmidt et al., 1995).

The C-fibre bearing group of neurones can be divided histochemically into two classes based on their ability/inability to express certain neuropeptides such as substance P

(SP) (Snider and McMahon, 1998). The peptidergic population expresses TrkA, the high-affinity tyrosine kinase receptor for nerve growth factor (NGF), and contains peptide neurotransmitters such as SP, brain-derived neurotrophic factor (BDNF) and calcitonin gene-related peptide (CGRP). This class of sensory neurones projects to the outermost region of the spinal dorsal horn (lamina I and outer lamina II), and terminates largely on spinal neurones that project to higher-order pain centres in the brain (Stucky et al., 2001)

The non-peptidergic population of unmyelinated C-fibres can be identified by the selective binding of the α-D-galactosyl-binding plant lectin IB4. This subpopulation expresses very few neurotransmitters and does not express the TrkA receptor. They are however responsive to and regulated by glial cell-line derived neurotrophic factor (GDNF) and they also co-express the c-ret tyrosine kinase receptor as well as the P_2X_3 receptor (Snider and McMahon, 1998). These neurones project mainly to the inner lamina II region which contains primarily local spinal interneurones (Stucky et al., 2001; Julius and Basbaum, 2001; Wiesenfeld-Hallin and Xu, 2001)

1.3.6 Pain mechanisms

Pain is initiated by nociceptors which respond to noxious stimuli. At the periphery these noxious stimuli (external or internal) activate specific nociceptive transducer receptor/ion channel complexes, leading to the depolarisation of the membrane.

Transducer proteins that respond to irritant chemicals have been identified and several are expressed exclusively in sensory neurones (VR1, DRASIC, P2X3) (McCleskey and Gold, 1999). Noxious heat transducers VR1(TRPV1) (vanilloid receptor/Transient receptor potential; vanilloid1) and VRL1(TRPV2,) are both members of the vanilloid receptor family (Benham et al., 2003). The noxious cold transducers TRPM8/CMR1 and TRPA1/ANKTM1 are also TRP channels in the Melatostatin subfamily (Clapham, 2003).

A transducer for noxious mechanical stimuli has yet to be identified. Studies on *Caenorhabditis elegans* indicates that it may belong to the MDEG (mammalian degenerin) ion channel family (Waldmann and Lazdunski, 1998).

The excitation of any or a combination of these primary transducers by a noxious stimulus leads to a local depolarization event and eventual production of an action potential assuming the stimulus is supra-threshold.

A second class of transmembrane channels which are found in the nociceptors, the voltage gated sodium channels (VGSC's) are responsible for the propagation of the action potential. These channels are activated by local depolarization events and allow an influx of sodium ions (Na^+) into the cell thus propagating the action potential along the axon towards the cell body located in the dorsal root ganglia (DRG) then on towards the central nervous system (CNS).

The arrival of the action potential at the central terminals of the nociceptive neurones, which terminate in the dorsal horn of the spinal cord initiates transmitter release. Fast excitatory synaptic transmission is mediated by glutamate release at the central terminals and their subsequent action ionotropic glutamate receptors, AMPA (α-amino-3-hydroxy-5-methyl-4-isoazoleproprionic acid), NMDA (N-methyl-D-aspartate) and kainite ligand-gated channels located on nociceptive dorsal horn neurones (projection and interneurones). Glutamate also binds to metabotropic receptors such as mGLUR and is recycled by glutamate-transport receptors which remove it from the synaptic cleft.

In the first instance spinal responses to non-tissue damaging noxious stimuli are mediated by glutamate acting on post-synaptic AMPA receptors in the dorsal horn. Repetitive or tissue damaging stimuli lead to the secondary activation of NMDA receptors leading to increased excitability of dorsal horn neurones (Dickenson and Sullivan, 1987).

Stimuli of greater intensities are associated with neurotransmitter release into the synaptic cleft from the central terminals of C-fibres. Substance P (SP) is one such transmitter released from dense core vesicles located on the central terminals of the nociceptive neurones. This SP binds to and activates NK-1 receptors on post-synaptic membranes leading to a sensitization of second-order sensory neurones.

At the dorsal horn, inhibitory processes mediated by other neurones most of which co-release glycine and γ-aminobutyric acid (GABA), may modulate the excitatory message en route to the brain for higher level processing. These stimulatory and inhibitory signals act in concert to encode the stimulus intensity and duration allowing for appropriate responses to be realised in the spinal cord and CNS.

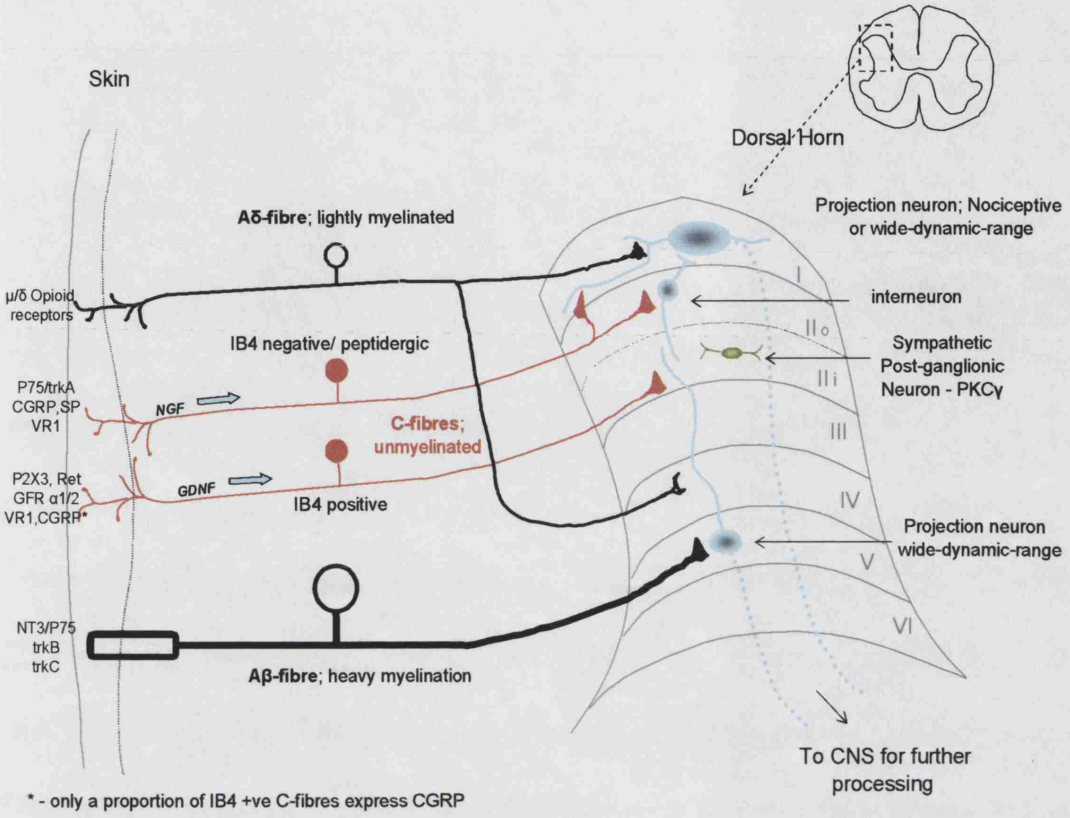


Figure 1. 2 Sensory pathways in the peripheral nervous system

1.3.7 Voltage gated sodium channels

Voltage gated sodium channels (VGSC) are important in providing the brief regenerative inward current that underlies the upswing of the action potential. They are therefore important in the propagation of action potentials elicited by the action of noxious stimuli on primary sensory neurones. There is a large molecular and pharmacological diversity in the members of the Na^+ channels present in the various tissues in mammals.

This diversity is derived primarily from α-subunit variability (see table 1.1) with additional contributions from the associated β-subunits. The α-subunit forms the pore of the channel. There are ten distinct VGSC α-subunits ($Na_v1.1$-1.9 & Na_x) approximately 260kDa in size, as well as four auxiliary β subunits (β1-4) (30-40kDa) so far identified (Blackburn-Munro and Fleetwood-Walker, 1999; Shah et al., 2000; Yu et al., 2003). Among the α-subunits it appears that $Na_v1.1$-$Na_v1.9$ are all related evolutionarily and can

be classed into one family (Na_v1) with Na_x forming another (Baker and Wood, 2001; Ogata and Ohishi, 2002).

Sodium channels are characterised by voltage-dependant activation, rapid inactivation, and selective ion conduction. Depolarization of the cell membrane in the vicinity of a sodium channel, allows the opening of the channel pore through which sodium ions passively enter the cell down their electrochemical gradient. These events lead to a further depolarization of the membrane and recruitment of additional Na^+ channels in the surrounding area. As the sodium equilibrium potential is reached an action potential is generated. Inactivation of the sodium channel occurs rapidly and is followed by a refractory period when the membrane becomes immune to further excitation.

Table 1. 1 Mammalian Voltage-gated Sodium channels

Channel	Old name	Gene	Chromosome (human)	Pharmacology (TTX)	Tissue expression	DRG level	KO Phenotype
$Na_v1.1$	Type I	SCN1A	2q24	TTX-s	CNS, PNS	Present	
$Na_v1.2$	Type II	SCN2A	2q23-24	TTX-s	CNS	Present	Lethal
$Na_v1.3$	Type III	SCN3A	2q24	TTX-s	CNS (embryonic)	Upregulated after axotomy	
$Na_v1.4$	SkM1	SCN4A	17q23-24	TTX-s	Skeletal muscle	Absent	
$Na_v1.5$	Cardiac,SkM2	SCN5A	3p21	TTS-r	Heart muscle	Absent	
$Na_v1.6$	Type IV, PN4	SCN8A	12q13	TTX-s	CNS, PNS, glia, nodes of ranvier	Abundant	
$Na_v1.7$	PN1	SCN9A	2q24	TTX-s	PNS, Schwann cells	Abundant	
$Na_v1.8$	SNS, PN3	SCN10A	3p21-24	TTS-r	PNS (sensory neurons)	Abundant	Partial analgesia
$Na_v1.9$	SNS2, NaN	SCN11A	3p21-24	TTS-r	PNS	Abundant	
Na_x	NaG	SCN6A, SCN7A	2q21-23	TTS-r ?	Heart, uterus, PNS glia, Smooth muscle	Present	Altered salt intake

Abbreviations; DRG, Dorsal Root Ganglia; KO, Knock-out; TTX-s, tetrodotoxin sensitive; TTX-r, tetrodotoxin resistant

VGSC's are glycoproteins and contain a central pore formed from the α-subunit. In many tissues this central ion conducting pore is associated with one or more accessory β-subunits. The $\beta1$ (or $\beta1A$) – subunit is associated with the α-subunit and by means of disulphide-bridge to the $\beta2$-subunit (Isom, 2000). β-subunits, which co-immunoprecipitate

with the cytoskeletal element ankyrin$_G$, are thought to play a rôle in modulation of the kinetics of the channel as well as determining the spatial location of the channel. They may also determine the level of channel protein expression (Baker and Wood, 2001; Isom, 2000; Malhotra et al., 2000).

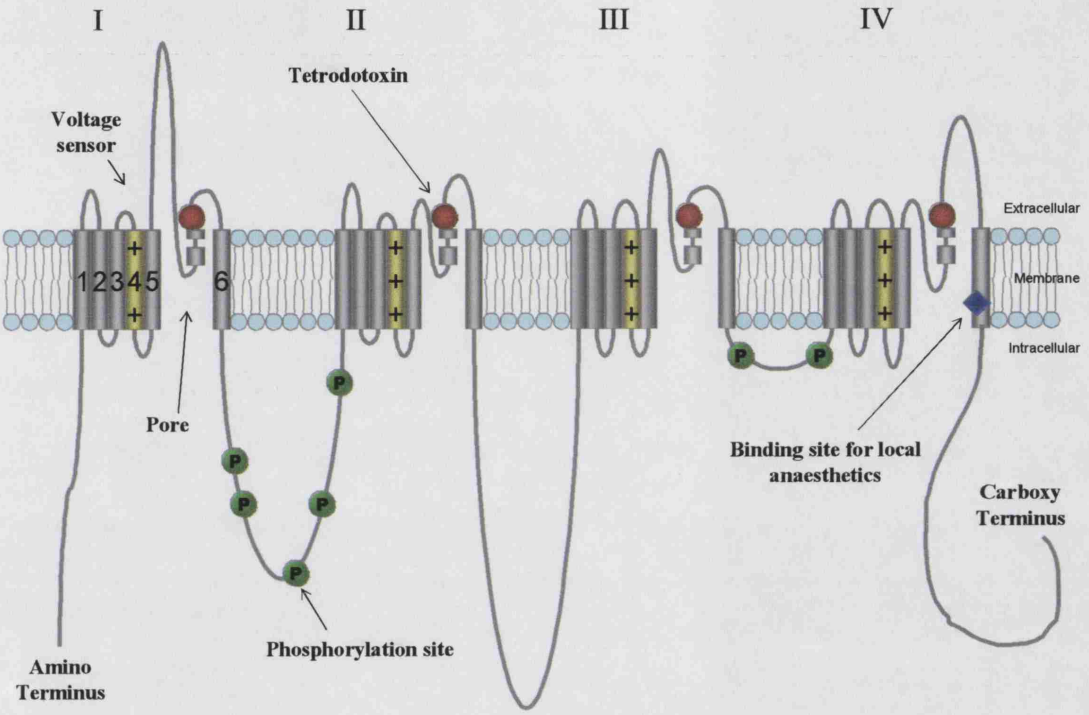


Figure 1. 3 Alpha-subunit of VGSC

The α-subunit illustrated above (Figure 1.3), is responsible for the formation of the ion-conduction pore and consists of four repeated homologous domains (I-IV), each containing six α-helical transmembrane segments (1-6), intracellular loops connecting the four domains, and intracellular amino- and carboxy-termini (Figure 1.2). The α-subunit plays a major role in the determination of the channel properties and can form a functional channel on its own. The fourth transmembrane segment, shown in yellow (the voltage sensor), is responsible for the voltage-gating property of the channel. This section of the protein moves in and out of the pore depending on the local charge environment, opening and closing the pore.

The discovery of a guanidinium toxin from puffer fish, termed tetrodotoxin (TTX) provided a useful way to characterise the different sodium channel isoforms. TTX binds to

and blocks specifically subtypes of Na+ channels at concentrations over the single nanomolar range. The toxin binding site has been determined to be the loop between the fifth and sixth transmembrane domain regions. Binding of the toxin occludes the channel pore and prevents its proper functioning.

An observation that action potential generation and propagation in skeletal muscle and many nerve fibres could be blocked by small concentrations of TTX, illustrated that the form which is expressed in these tissues is sensitive to TTX. These channels and others like it have been termed TTX-sensitive based on their response to tetrodotoxin, and the currents that they generate have been termed TTX-s currents as a result.

Interestingly, there are a number of α-subunits (Na_x, $Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) which remain unaffected by micromolar levels of Tetrodotoxin and continue to generate a current (termed TTX-r current). In addition to the heart, where $Na_v1.5$ is found, these resistant currents have been found to be present in a high proportion of small-diameter (<25μm across) DRG neurones. These small diameter neurones seem to be predominantly associated with C-and Aδ- type fibres which have been proposed to transmit nociceptive information. These TTX-r currents remain operable at concentration that block TTX-s currents by >99% (reviewed in Baker, M.D. et al, 2001).

A hydrophobic aromatic residue within the first domain P-loop of the Na+ channel amino acid sequence is crucial for TTX-binding. The P-loop region is involved in the outer lining of the pore and contributes the ion selectivity of the final channel. Tetrodotoxin binding at this site therefore occludes the pore and physically blocks the entry of ions into the pore. TTX-r channels contain a hydrophobic residue at this point and therefore remain immune to higher concentrations of TTX. Studies on the TTX-r channel $Na_v1.8$ (SNS) have shown that by converting the hydrophobic serine at position 356 into a phenylalanine, a four-order of magnitude increase in TTX sensitivity can be induced in the channel (60μm-2nM) (Sivilotti et al., 1997).

1.3.7.1 Sodium channel distribution

The distribution of the VGSC's varies both spatially and temporally. Disturbances in the proper localisation and or expression pattern of these channels are thought to play a role in the development of chronic pain states. Generally, sodium channels are clustered

together with receptors in specific areas of neuronal membrane, for example, the neuromuscular junction and myelinated nerves which show heterogeneous distributions of their Na^+ channel complement. Furthermore the association of the α-subunits with β-subunits is unique to neuronal Na+ channels.

The four β-subunits are unique among ion channel subunits so far studied. They contain several immunoglobulin type domains on their extracellular domains. These are similar to those found in many adhesion molecules (Isom, 2000).

The α-subunit isoforms, which are mainly responsible for the channel characteristics, have distinct patterns of development and localization in the nervous system, skeletal and cardiac musculature (Table 1.1). $Na_v1.4$ is expressed in adult skeletal muscle replacing $Na_v1.5$ which appears in embryonic muscle and in denervated skeletal muscle. In the adult, $Na_v1.5$ is normally found in the heart muscle where it undergoes slow inactivation and displays a TTX-resistant current (IC50 1.8μM)(Waxman et al., 1999).

$Na_v1.1$ is well expressed in the adult CNS; Purkinje cells, hippocampal pyramidal cells, spinal motor neurones and other neuronal somata, and in limited quantities in DRG. $Na_v1.2$ is predominantly expressed in the CNS. Nav1.2 Knock-out mice are perinatal lethal, illustrating the importance of this channel in development. $Na_v1.2$ also plays a rôle in the developing PNS, where it is replaced by $Na_v1.6$ at maturity. $Na_v1.6$ occurs mainly in the DRG, glia and peripheral axons particularly at the nodes of Ranvier as well as the adult CNS (Baker and Wood, 2001; Waxman et al., 2002).

$Na_v1.3$ is found in the embryonic CNS and DRG but disappears at adulthood, only reappearing following neuronal insult. This suggests that it may play a part in the development of neuropathic pain (Novakovic et al., 1998).

The remaining members of the first family of α-subunits ($Na_v1.7$, $Na_v1.8$ and $Na_v1.9$) are all well expressed in DRG. $Na_v1.7$ is expressed in DRG (all diameter neurones), brain and spinal cord but appears to be absent from the node of Ranvier where $Na_v1.6$ is well expressed. $Na_v1.7$ is also regulated by NGF and exhibits fast inactivation kinetics, sensitive to TTX with an IC50 of 2nM (Wood et al., 2002).

Both $Na_v1.8$ (SNS) and $Na_v1.9$ (NaN) are well expressed in small diameter neurones. The kinetics of $Na_v1.8$ in particular have been used to link this channel with the specific processing of nociceptive information and involvement in the ectopic discharges

believed to underlie neuropathic pain symptoms. Both of these subtypes are resistant to TTX ($Na_v1.8$; IC50 >100μM, $Na_v1.9$; IC50 > 1μM) (Dib-Hajj et al., 2002; Akopian et al., 1999).

The remaining voltage-gated sodium channel α-subunit, Na_x, is sufficiently different to be placed in a family by itself. It is expressed in the heart, uterus, smooth muscle, PNS and glia. Reviewed in (Baker and Wood, 2001; Wood et al., 2002; Ogata and Ohishi, 2002; Goldin, 2001; Novakovic et al., 2001).

1.3.8 Clinical pain – inflammatory, neuropathic and cancer related pain

In contrast to acute pain processes, chronic pain states are initiated in response to tissue damage/inflammation, nervous damage, and cancer or may appear spontaneously. These pain states are often accompanied by allodynia and hyperalgesia. The development of inflammatory or cancer pain (associated with ongoing inflammation) may signal that ongoing protection is required during the healing phase, however many chronic pain conditions including those associated with neuropathic injuries or some cancer pain conditions do not serve any useful purpose and it is difficult to imagine evolutionarily advantageous reasons for it (e.g. phantom limb pain).

Many processes associated with acute pain as described earlier become modified or augmented leading to the generation of these clinical pain states. Changes at the periphery as well as centrally in the spinal cord/CNS are implicated in the development of chronic pain. These changes are peripheral- or central-sensitization related.

1.3.8.1 Peripheral Sensitization

Peripheral sensitization leads to increased excitability in nociceptors. The level of depolarisation required to elicit an action potential is decreased allowing previously sub-threshold stimuli to cause a discharge. Exposure of the terminal to many agents leads to peripheral sensitization (Julius and Basbaum, 2001). Many inflammatory agents (PGE2; prostaglandin E2, 5-HT; bradykinin, epinepherin, adenosine) exhibit a sensitizing effect at nerve terminals (Wood and Docherty, 1997).

Neurotrophic factors released during tissue damage or by inflammatory cells can sensitize the terminals directly or indirectly (Andreev et al., 1995; Kerr et al., 1999). This sensitization is limited to the affected area and involves the phosphorylation of $Na_v1.8$ (England et al., 1996b) and possibly VR1 by activated protein kinase A (PKA) or protein kinase Cϵ (PKCϵ) (Aley and Levine, 1999) .

Both the phosphorylation of, and the action of guanine nucleotide binding proteins (G proteins) on the transducer molecules and VGSC's can alter their properties, including activation threshold and rate of activation/inactivation(Bevan and Storey, 2002). The large number of complementary molecules which converge on these two pathways suggests that elimination of one component may not be sufficient to eliminate peripheral sensitization.

In addition to post-translational changes, transcriptional and/or post translational changes mediate peripheral sensitization. Changes in the expression of TRPV1 can be observed during inflammation, exacerbating heat hyperalgesia, have been shown to be mediated at the peripheral terminals through increased expression and intracellular channel shuttling mechanisms (Ji et al., 2002b).

1.3.8.2 Central sensitization

In addition to glutamate release at the central terminal of nociceptive neurones(Young et al., 1998; Niederberger et al., 2003), neuropeptides (SP) (Cao et al., 1998; Couture et al., 2003), and neurotrophic factors (BDNF) (Pezet et al., 2002a) are released into the dorsal horn. These act as co-transmitters and induce long-lasting changes in spinal excitability termed central sensitization (Woolf, 1983). NMDA receptors are centrally implicated in this process (South et al., 2003).

1.3.8.2.1 Mechanisms of central sensitization;

a) Wind-up

A reversible activation-dependant change in dorsal horn neurones is termed wind-up. This process occurs due to a temporal summation of sub-threshold inputs, release of neuromodulators, and activation of voltage-gated calcium currents together with the

removal of the Mg^{2+} blockage of NMDA channels in the dorsal horn leading to the generation of an action potential (Dickenson and Sullivan, 1987).

The behavioural correlate has been observed where repeated stimulation with identical noxious stimuli results in progressively greater responses (Straud et al., 2003).

b) Dorsal horn LTP

LTP or long term potentiation was first observed in the CNS. Long term synaptic priming could be achieved following a brief high-frequency conditioning stimulus. A similar phenomena was described in the spinal cord and is recognised as a mechanism implicated in central sensitization (Ji et al., 2003).

In the dorsal horn, frequencies around 100Hz have elicited LTP lasting tens of minutes, however under physiological conditions such high frequency impulses do not occur with regularity hence the role of such systems is unclear. Intracellular Ca2+ levels particularly in NK-1 expressing lamina one dorsal horn neurones may be implicated in this mechanism (Ikeda et al., 2003).

c) Transcriptional changes

The stimuli associated with the sensitization process recruits large numbers of diverse protein kinases such as PKA (Aley and Levine, 1999), PKC and ERK(Pezet et al., 2002b; Ji et al., 2002a). These activated molecules in turn phosphorylate and activate a wide variety of cellular transcription factors which enhance the expression of a diverse range of nociceptive-related genes including the immediate early genes c-fos (Hunter et al., 1995; Gillardon et al., 1997) and COX-2 (Vane et al., 1998) amongst others. The upregulation of a wide range of proteins augments the nociceptive pathway and results in long-term alterations of the sensitivity of the nervous system.

1.3.9 Inflammatory pain

Inflammatory pain including the changes in the sensitivity of nociceptors associated with inflammation is a common consequence of tissue injury. Burns abrasions, infections

and cancer can all lead to the generation of pro-inflammatory signals and mechanisms including neurotrophin release (Jaggar et al., 1999; Ji et al., 2002b), mast-cell degranulation (Rice et al., 1998), tissue acidosis, plasma extravasation amongst others (Andreev et al., 1995). Inflammatory pain is characterised by this concert of mechanisms and begins within minutes of the insult and lasts generally until healing has been completed (Dmitrieva et al., 1997).

1.3.9.1 Pathophysiology of inflammatory pain

Inflammation is linked to tissue damage including burns, laceration and tumourogenesis. This leads to the seepage of intracellular contents into the extracellular environment. The recruitment of inflammatory mediators and the production and release of a number of neuroactive agents by inflammatory and non-inflammatory cells, including ions (K+ and H+) (Waldmann and Lazdunski, 1998), kinins such as bradykinin (Couture et al., 2003), amines (histamine and 5-hydroxytryptamine (5-HT)), purines (ATP) (Paukert et al., 2001), nitric oxide, prostanoids such as PGE2 (Ito et al., 2001), cytokines (IL-1, TNF-α) (Woolf et al., 1997) and growth factors such as NGF (Jaggar et al., 1999; Kerr et al., 2001) act in concert and produce rubor (redness), calor (heat) and oedema.

Some of these inflammatory factors (e.g. 5HT) are sufficiently active to affect the peripheral sensory endings of the nerve, generating inward currents and sensory inflow. However most act by modulating the sensory neurones rather than activating them. These changes include post-translational changes in both the peripheral terminal and the dorsal horn. This may be followed by longer-term transcription-dependant changes in the DRG and spinal cord, induced by the retrograde transport of inflammatory mediators such as NGF to the cell bodies (Jaggar et al., 1999; Ji et al., 2002a).

Tissue injury results in the release into the extracellular fluid, of inflammatory mediators from damaged cells including kinins. Members of the kinin family have between 9-11 amino acid residues in their peptide chains and count bradykinin, kallidin, T-kinin and their active metabolite amongst their number. These molecules undergo rapid metabolic degradation by amino-, carboxy- and endo-peptidases found in tissues and biological fluids (Couture et al., 2003), as a consequence their activity on inflammation occurs mainly in the acute phase. Bradykinin plays an important role in the early

development of the post-traumatic pain situation (Burch and DeHass, 1990). When bradykinin is administered subcutaneously to human subjects, it produces pain, inflammation and hyperalgesia (Meller and Gebhart, 1992). Bradykinin sensitises small cells to heat via PKA (protein-kinase A). Additionally, bradykinin and its congener kallidin, together with their degradation products, des-Arg bradykinin and des-Arg-kallidin have complex effects on primary sensory neurones including direct activation via the B1/2 receptors present on both neuronal and non-neuronal cells (Bley et al., 1998).

Studies using the B2-high affinity bradykinin receptor antagonist, Bradyzide, have blocked inflammatory hyperalgesia in animals models of inflammation (Burgess et al., 2000). Conversely, the B1 agonist has been found to specifically produce pain during inflammation but not neuropathic conditions, suggesting that enhanced expression of the B1 receptor or sensitisation of this receptor is required (Dray and Perkins, 2003).

The B2-receptor mediated pathway has been proposed to act during the acute phase with the B1-receptor pathway (activated by bradykinin metabolites) acting longer term. The half-life of des-Arg bradykinin is 4- to 12- fold longer than that of bradykinin (Decarie et al., 1996b) and studies using the carrageenan model of inflammation have shown that the degradation product des-Arg were 1.3- to 5-fold greater that bradykinin itself in the inflamed paw (Decarie et al., 1996a; Burch and DeHass, 1990).

Cytokines are also present in the inflammatory soup associated with the inflammation injury. They act to initiate and maintain inflammatory disease as mediators of cell-cell interactions. Cytokines also act directly on neurones in a similar manner to kinins. The indirect action of cytokines, through prostaglandin release appears to be the more important pathway. During the acute phase of the inflammatory response, cytokines act to induce sensitisation of the sensory afferents through receptor-associated kinases and channel phosphorylation and in the later stages promote transcriptional up-regulation of receptors leading to pain maintenance (Opree and Kress, 2000).

Prostaglandins (PG) are a group of Arachidonic-acid (AA) derivatives containing a cyclopentane ring and act as local hormones in a number of physiological conditions including inflammatory pain (Ito et al., 2001). Unlike substance P (SP) or glutamate, the AA precursor of PGs are not stored in vesicles but held in the membrane by phospholipids ready to be released by the action of phospholipase A_2. The generation of PGD_2, PGE_2 $PGf_{2\alpha}$ and PGI_2, collectively known as prostanoids, occurs in the arachidonic acid cycle.

The development of inflammation can be blocked though the interference of the production of PG's. This can be accomplished chemically by the administration of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs), which act to stop PG production by peripheral tissues (Vane, 1997). In particular, these drugs act upon the rate-limiting enzymes of the arachidonic acid pathway, Cyclooxygenase-1 (COX-1) and COX-2. These are both isoforms of the PGH synthase enzyme which catalyses the initial two reaction leading from the precursor arachidonic acid via PGG_2 to PGH_2 the intermediary molecule leading to the production of most prostanoids (Vane et al., 1998).

PGs, mainly PGE_2 and PGI_2 are rapidly produced following tissue damage or inflammation by most cells at the site of injury in response to noxious sensory stimuli. This rapid activity makes them important in mediating the acute pain associated with inflammation (Ito et al., 2001). In addition, the COX-2 form of the PGH synthase appears to function as an inducible isoform and has been shown to be up-regulated in several animals models of inflammation (Siebert et al., 1994).

Prostaglandins, which are rapidly produced at the inflammation site, by a wide variety of cells, sensitise nociceptors. Intradermal injection of PGE_2 and PGI_2 is thought to act directly on primary afferent terminals of sensory neurones using the c-AMP second messenger system (England et al., 1996b). This produces a mechanical hyperalgesia in the injected site that is PKA dependent as shown by the use of PKA inhibitors (Aley and Levine, 1999). This PKA then phosphorylates a wide variety of receptors on the peripheral terminals of sensory afferent (such as $Na_v1.8$) and alter their sensitivity to noxious and non-noxious stimuli (Aley and Levine, 1999).

Intrathecal administration of PGE2 has also been reported to induce allodynia in mice (Ito et al., 2001). The induction of this allodynia was within 5 minutes of application and lasted for at least one hour post-injection. Block of NMDA, AMPA receptors and inhibitors of NO-synthase (NOS) all blocked this allodynia but NK-1 receptor antagonists were unable to do so.

The neurotrophin family, including nerve growth factor (NGF), brain-derived nerve factor (BDNF) and glial-derived neurotrophic factor (GDNF) make significant and long-lasting contributions to the changes in neuron sensitivity during inflammation (Lewin and Barde, 1996).

NGF, in particular has been widely studied and implicated in the peripherally activated mechanisms following inflammation. In humans subcutaneous NGF produces cutaneous hyperalgesia at the injection site and widespread deep pain which persists for many days (McMahon and Bennett, 1999). The peripheral application of NGF has also been shown in animals to induce the development of inflammatory pain-like behaviour together with hyperalgesia and allodynia. This suggested that NGF has a peripheral site of action.(Andreev et al., 1995; Thompson et al., 1995)

NGF is present in the skin and is upregulated in inflammatory conditions (Aloe et al., 1992). Studies using IgG sequestration molecules (McMahon et al., 1995) or anti-NGF antibodies (Lewin et al., 1994) to remove the NGF produced on inflammation from mast cells diminishes the hyperalgesia associated with inflammation.

NGF also stimulates the upregulation of a wide range of neurogenic peptides (SP and calcitonin-related gene peptide; CGRP) (Woolf et al., 1994) and affects the expression of various channels including $Na_v1.8$ (Fjell et al., 1999; Kerr et al., 2001), on neurones that are important in pain transmission. The effects take place several hours post exposure to NGF and contribute to the establishment of changes in the dorsal horn leading to central sensitisation of the system (Lewin et al., 1994).

In addition to classic trophic action as exemplified by NGF, another member of the neurotrophic family, BDNF has been implicated in synaptic manipulation. BDNF is upregulated by NGF, and by inflammation, and is retrogradely transported to the central terminals of the efferent nerve. Here it is stored in vesicles and is released in an activity dependant manner, leading to the modulation of synaptic activity (Michael et al., 1999; Cho et al., 1997b).

Neurogenic substances such as the tachykinin SP, which are stored and released along with glutamate from vesicles in the central terminals of small diameter peptidergic sensory neurones in the dorsal horn also contribute to the inflammatory response. Distal terminals of small-diameter neurones at the periphery also release CGRP and SP, leading to the wheal and flare associated with tissue damage and inflammation. In peripheral tissues SP acts to produce plasma extravasation and CGRP acts on arterioles to produce vasodilation. A synergistic cooperation between this pair of molecules has been observed (Brain and Williams, 1998).

Substance P degranulates mast cells to produce histamine release, induces release of PGE2 and collagenase from synoviocytes and may stimulate the release of cytokines from macrophages (reviewed in (Maggi, 1997)). Disruption of the preprotachykinin A gene (PPT-A), a precursor molecule for SP, has demonstrated the importance of SP in mediating medium to severe noxious stimuli transmission, in concert with glutamate, and also its central role in extravasation (Cao et al., 1998).

In addition to these post-translational changes, an alteration in the expression of effector molecules in the DRG and dorsal horn occurs upon inflammation. There are two main pathways;

1. The CREB (cAMP-responsive element-binding protein) and
2. Ras/MAP (mitogen-activated protein) kinase pathways that are responsible for regulation of gene transcription in the nucleus.

Activity-dependant activation of the CREB pathway with repeated peripheral stimulation, leads after a delay of several hours to an alteration in the transcriptional activity of the neuron and an increase in the transport of mature proteins to the terminals.

Alternatively, activation of the small G-protein Ras and Map-kinase after inflammation/neuropathic injury alters gene expression in the dorsal horn. Phosphorylation of various transcription factors including c-Myc, Elk-1 and c-Jun is mediated by this pathway and results in the translocation of MAPK to the nucleus where it increases the expression of genes via Elk-1 and CREB (Woolf and Costigan, 1999).

The transcriptional changes which accompany central sensitisation may induce the expression of nociceptor proteins such as SP in non-nociceptive neurones (Zimmermann, 2001; Brain and Williams, 1998). An example of this is noted in neuropathic injury where large diameter Aβ-fibres begin to synthesize SP which is then available for release at the dorsal horn upon non-nociceptive activation of the Aβ terminal by normally non-noxious stimuli such as light touch (Zimmermann, 2001).

1.3.9.2 Models of Inflammation

Due to the complex nature of the inflammatory condition, several *in vivo* models have been developed to allow the study of the mechanisms involved (Dmitrieva et al.,

1997; Buerkle et al., 1999). Many of these require the introduction of a chemical agent such as formalin(Hunskaar and Hole, 1987), carrageenan (Burch and DeHass, 1990) or NGF (Lewin et al., 1994; Jaggar et al., 1999) into the animal tissue to elicit an inflammatory response.

The development of thermal hyperalgesia and mechanical allodynia over the course of the inflammatory response is then tracked by applying a suitable stimulus, for example heat from a directed infra-red source to determine pain thresholds and thermal hyperalgesia or using hairs to apply a measurable force to test for allodynia.

The utilisation of these models, together with application of agents to reduce gene products of interest by controlled gene deletion in the animal or application of pharmacological agents has lead to the gradual dissection of the inflammatory process (Cummins et al., 2000). The development of technologies allowing the modification of genes implicated in inflammation, in functionally defined subsets of neurones, will aid in the understanding of inflammatory pathways.

1.3.10 Neuropathic pain

Neuropathic pain results from direct involvement of the nervous system in the pain generation process and is classed as a pathological condition (Suzuki and Dickenson, 2000). This deviation from the normal nociceptive function of the nervous system can be caused by direct injury to the peripheral or central nervous system through;

a) Mechanical nerve injury – e.g. vertebral disc hernia

b) Neuroma

c) Metabolic disease – e.g. diabetic polyneuropathy

d) Viral attack – e.g. Herpes Zoster (HSV), human immunodeficiency virus (HIV)

e) Neurotoxicity – e.g. tuberculosis or chemotherapy damage

f) Inflammatory and/or immunogenic changes – e.g. Multiple sclerosis

g) Nervous system focal ischemia – e.g. thalamic syndrome

h) Multiple neurotransmitter system dysfunction – e.g. complex regional pain syndrome (CPRS)

1.3.10.1 Pathophysiology of neuropathic pain

Both central and peripheral changes in the nervous system have been implicated in the development of neuropathic pain conditions. Following nerve transection there is the development of a neuroma at the proximal end of the cut nerve (Zimmermann, 2001). This neuroma consists of nerve sprouting at the site of injury.

During this regeneration event, multiple unmyelinated sprouts issue from each transected axon. These changes are accompanied by intense and spontaneous nerve discharges, particularly from C-fibres. After only a few hours mechanical stimulation at the site of injury can elicit discharges and a few days after the insult spontaneous c-fibre mediated discharges can be observed (Ochoa et al., 1982). The novel nerve sprouts also show sensitivity to noxious heating (Zimmermann, 2001)

These observation together with other evidence from the study of the transected ends of the nerve have demonstrated that the abnormal behaviour of the axons is maintained by the unusual accumulation of various ion channels at the proximal end, together with the novel expression of some channel subtypes due to the nerve injury (Devor et al., 1993; England et al., 1996a).

In particular, VGSC's have been shown to play an important role in this process (Devor et al., 1993; England et al., 1994). The TTX-sensitive channel $Na_v1.3$ has been shown to be upregulated with nerve transection (Novakovic et al., 1998), whilst immunochemical studies have demonstrated that the TTX-resistant forms $Na_v1.8$ and $Na_v1.9$ are down-regulated but accumulate at proximal end of the nerve at the injury site (Coward et al., 2000; Gold et al., 2003). The interplay of the various subtypes of sodium channels is important in determining the response thresholds and firing dynamics of the axon, therefore these changes play an important role in the modulation of the nervous system after nerve insult.

Experiments with explanted nerve preparations have shown that regenerating axonal sprouts develop chemosensitivity within days to substances such as bradykinin or SP (Zimmermann, 2001). In experiments with cat neuroma preparations, stimulation with bradykinin stimulated a quarter of the C-fibres to generate long-lasting discharges, but no such observation was noted in the A-fibres (Zimmermann, 1985) suggesting selective expression of receptor on particular axon subtypes.

The normal functioning of a nervous system is also dependant on a wide range of neurogenic factors (NGF, BDNF) which are either target derived or centrally produced from the cell bodies in the DRG (Bennett et al., 1996; Miki et al., 2000). With the transection of the nerve these transport routes are disrupted leading to the abnormal expression of proteins and inappropriate discharges from the nerve. These changes can be mimicked by chemical sympathectomy methods which exploit the ability of cytostatic drugs (vincristine or taxol) to block these axonal transport routes, leading to neuropathic-like conditions (Lindsay and Harmar, 1989).

Nerve injury affects not only axons but also the associated Schwann cells which produce the myelin sheaths protecting the axon. It has been observed that with nerve injury the Schwann cell distribution and function is altered with a dramatic increase in growth factor synthesis. This together with the concurrent down-regulation of myelin production at the injury site, there follows a redistribution of sodium channels such as $Na_v1.2$ and $Na_v1.6$ which are normally associated with the nodes of Ranvier only, to a more widespread occurrence along the length of the axon (Hinson et al., 1997). This may have important consequences on action potential generation.

Central changes have also been shown to be important in the development of neuropathic pain (Zimmermann, 2001). The absence of target derived neurotrophic factors such as NGF leads to changes in the dorsal horn chemistry in the area served by the damaged nerve. The reduction in these signals together with abnormal c-fibre firing patterns may act in concert to change the dorsal horn neuron phenotypes such that they become more prone to firing (sensitised), experience wind-up and/or long-term potentiation (LTP) (Ji et al., 2003). An increased responsiveness to sympathetic transmitters, may also underlie the chronic burning pain observed in neuropathic patients (Yao et al., 2002).

It has also been observed that the descending spinal inhibitory pathways in neuropathic animals are impaired. This together with a decreased efficacy of the endogenous spinal opioid system seen following peripheral nerve section in rats and dramatic NMDA-receptor-dependant decrease in opioid analgesia following nerve injury suggests that there is impairment in the normal inhibitory pathways in neuropathic conditions leading to increased nociception (Zimmermann, 2001).

1.3.10.2 Models of Neuropathic pain

Most of the animal models which have been developed to address the question of neuropathic pain and its causes have utilised direct injury to peripheral nerves to induce the condition. Four different injuries to the nerve are commonly applied to elicit the neuropathic condition in animals

a) Total transection of a peripheral nerve such as the sciatic (Wall et al., 1979)
b) partial transection (about 50%) of the nerve (Seltzer et al., 1990)
c) Chronic constriction of the nerve with a series of loose ligatures leading to a reduction of the original nerve diameter by approximately half (CCI model) (Bennett and Xie, 1988)
d) Tight ligation of the nerve by ligatures (SNL model) (Kim and Chung, 1992)

These models produce similar, though not entirely identical effects and the reproducibility of the neuropathy induced by a particular model may be highly variable. Notwithstanding this caveat, there are a number of behavioural observations which have been linked to the development of the neuropathic condition in animals.

The behaviour known as autotomy, where the animal self-mutilates the area innervated by the affected nerve is one measure of the condition. However it has been noted that other non-neuropathic conditions such as skin inflammation can elicited injuries seen as correlative of this type of behaviour and therefore care must be taken in its assessment (Corderre et al., 1986).

Measurements of mechanical and thermal responses to both noxious and non-noxious stimuli can also be taken to assess the development of hypersensitivity and allodynia in these models. Used in conjunction with specific ablation or upregulation of putative neuropathic-related genes we have a powerful experimental system for the elucidation of the pathways leading to the generation of ectopic discharge and neuropathic pain.

1.4 Aims of thesis

The aims of this thesis were two fold. Firstly, to identify suitable methods to allow the efficient and selective transduction of defined subsets of sensory neurones (in particular nociceptors). Secondly, the use of these techniques to examine the involvement of putative nociceptive genes (identified using *in vitro* protocols) in animal models of nociception and pain was undertaken. These results should help to validate analgesic targets for pharmacological intervention.

The use of antisense protocols, recombinant HSV vectors and promoter-limited Cre-expressing transgenic lines was explored and quantification of the number and specificity of transduced neurons will be done. In particular methods which preferentially targeted small diameter neurons (which constitute thhe majority of nociceptors) were sought. The establishment of an approach which achieves both a high level of transduction of nociceptors coupled with a low level of transduction of non-nociceptors (as well as other non–neuronal tissues) was desirable.

Three diverse techniques were trialled. The use of antisense molecules has already had some success in achieving *in vivo* modification of genes (Zhu et al., 1997; Khasar et al., 1998; Dorn et al., 2001; Lai et al., 2002). The ability of antisense molecules which have been previously demonstrated to achieve knock-down of a $Na_V1.8$ accessory protein in vitro (p11) (implicated in the shuttling of the channel to the membrane where it can function) (Okuse et al., 2002) was extended *in vivo*, to determine the relative contribution of this pathway to the generation and maintenance of pain, (particularly in inflammatory conditions). This result would help to validate p11-$Na_V1.8$ interaction as a valid pharmacological interevention target to address inflammatory pain.

The development of recombinant HSV vectors has provided a useful method of preferentially targeting neurones. The biology of HSV itself has been proposed as an advantage of these vectors in achieving specific and high level manipulation of the peripheral and central nervous system (Lilley et al., 2001a). In particular new vectors have been developed to achieve high level and specific transduction of sensory neurones in the PNS (Palmer et al., 2000). These vectors will be investigated to determine their potential and the number of nociceptors successfully transduced will be assessed. As HSV vectors allow transduction of neurones to occur following relatively non-invasive introduction

methods, attempts will be made to overcome any shortcomings encountered through their use.

The use of transgenic animals is well established for the study of pain (Croll et al., 1999). However, the development of knock-out and over-expressing strains for a particular gene under investigation is both expensive and time consuming. Recent work has therefore concentrated on the development of a series of animals which display tissue and temporally restricted Cre-recombinase expression (Tsien et al., 1996; Metzger et al., 1992). These animals can be used to characterize the requirement of a gene in pain pathways. The central role of nociceptors in the pain pathway has lead to the search for a nociceptor-limited Cre-recombinase expressing strain. Recent attempts have identified two strains which may prove useful ((Hatano et al., 1997b) and (Stirling et al., 2003)). The first strain expresses Cre-recombinase under the control of the Ncx promoter. The Ncx gene belongs to the homeobox family, and has been identified as efficiently driving expression of Cre-recombinase in neural-crest derived tissue in embryos (Hatano et al., 1997b). The expression pattern will therefore be investigated to determine its suitability in driving nociceptor-specific excision in dorsal root ganglia (DRG). DRG are developmentally related to, and contain a proportion of cells derived from the neural crest. In order to determine this, the Ncx-Cre expressing strain will be crossed with the Rosa-26R strain (Zambrowicz et al., 1997; Soriano, 1999) which contains an inactive form of the β-Galactosidase gene which can be activated by the action of Cre-recombinase.

The development of a $Na_V1.8$-limited Cre-expressing line has been recently achieved (Stirling et al., 2003). This strain has been demonstrated to achieve a high level of nociceptor specific gene excision using a reporter strain (Stirling et al., 2003). The utility of this strain has been further supported by the normalcy of many behavioural test (including several pain models) when compared with control animals.

The $Na_V1.8$ –Cre conditional mouse strain was used to specifically ablate an important neurotrophin (BDNF) to further define the contribution of this neurotrophin in the establishment and maintenance of pain states, particularly inflammatory conditions. Previous work has implicated this neurotrophin in a wide variety of pain pathways and processes (Siuciak et al., 1995; Pezet et al., 2002c; McMahon and Bennett, 1999). However, limitations in available technologies prevented the confident explanation of the source of the BDNF responsible for these observations. The use of this BDNF-limited

cross was used to more completely understand the role of the BDNF released from C-fibres.

Taken together these approaches to manipulate gene expression in pain pathways should provide useful information on potential new targets for analgesic drug development.

Chapter 2: Development of a Cre-recombinase expressing HSV vector to specifically target sensory neurones in dorsal root ganglia.

2.1 Synopsis

The tropism of HSV-derived vectors for neurones has been well documented. At present, efforts have focussed mainly on the development of these systems to target CNS neurones. However HSV can also move retrogradely along nerve fibres and can be used to introduce genes into neurones of the peripheral nervous system even when administered at the peripheral nerve terminals.

The manipulation of sensory neurones and in particular nociceptors of the PNS was investigated through the use of modified HSV vectors. The development of a Cre-expressing HSV vectors targeted at the DRG is described in this chapter. To achieve the successful manipulation of DRG neurones, HSV-derived viral vectors expressing both Cre-recombinase as well as GFP were introduced into Rosa-26R reporter mice (see chapter 4) via injections of various titres of vectors either directly into the sciatic nerve or into the footpad. The expression of functional β-galactosidase was used to determine the infection rate.

The type and number of transduced neurones in the DRG from both the ipsilateral and contralateral tissue (to the injection site) were quantified. This was done for two timepoints i.e. one week and one month post-introduction of the vector. The suitability of this vector to drive sensory neuron (particularly nociceptors) gene ablation was determined.

It is hoped that the use of HSV vectors will allow temporally as well as spacially controlled gene ablation. This method should circumvent the problems encountered when genes are deleted unconditionally during development, a common complication with other protocols such as unconditional transgenic animals.

2.2 Introduction

The HSV vector system has been identified as a tool which offers the ability to manipulate gene function in sensory neurones located in the dorsal root ganglia (DRG) (Coffin et al., 1996; Ecob-Prince et al., 1995; Dobson et al., 1990; Palmer et al., 2000).

Several characteristics of HSV biology are exploited in effecting gene manipulation when using HSV-based vector systems. Pertaining specifically to neuron infection several features stand out;

1 Non-dividing cells (such as neurones) can be efficiently transduced and support expression of transgenes ((Lachmann and Efstathiou, 1997).
2 Several endogenous HSV promoter sequences have been characterised which have been shown to allow efficient, long-term expression of transgenes (Arthur et al., 2001; Palmer et al., 2000).
3 Approximately half of the 84 known HSV-1 genes are non-essential for virus growth allowing large insertion sequence sizes (Krisky et al., 1998).
4 Recombinant strains can be readily isolated and grown to high titres without contamination from wild-type virus (Ozuer et al., 2002).
5 Retrograde transport of the vector along microtubules to the cell body allows the introduction on the virus at peripheral sites without the need for highly invasive surgical techniques such as direct DRG injection (Smith and Enquist, 2002; Palmer et al., 2000).
6 Highly disabled vectors can be produced with essential gene products delivered *in trans* during culture to provide vectors with improved cytotoxicity profiles (Burton et al., 2002; Lilley et al., 2001b).

2.2.1 Development of HSV-based vector systems

HSV is a large DNA virus characterised by its ability to enter a lifelong latent state in neurones from which reactivation can occur intermittently. The two problems encountered when developing HSV-based vector systems are;

1 Disabling the virus to reduce the pathogenicity whilst retaining its infective profile.

2 Maintenance of transgene-function during HSV latency and concurrently suppressing the expression of viral genes which are cytotoxic.

Due to the large 154kb HSV-1 genome, manipulation by conventional cloning techniques is impossible. A series of shuttle cassettes have been developed (Coffin et al., 1996; Glorioso et al., 1995; Fink et al., 1996) which can be manipulated to include transgenes as required and then inserted into the HSV-1 genome (Fig 2.1) by homologous recombination during growth in eukaryotic cells in culture.

Figure 2. 1 Herpes simplex virus genome

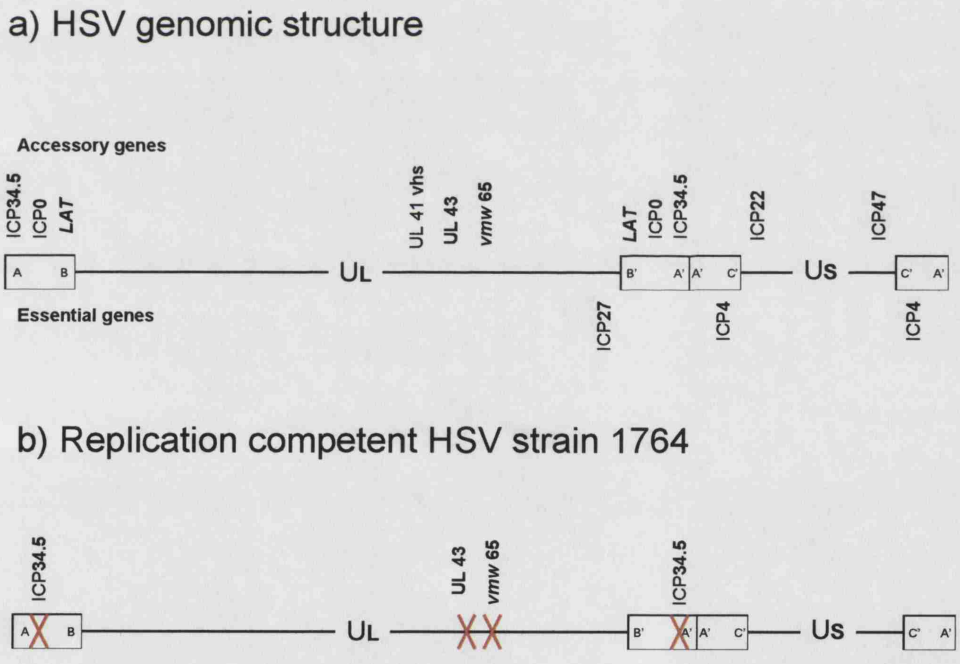


Figure 2.1: a) the HSV genome illustrating the two unique regions U_L and U_S flanked by inverted terminal repeat sections. The genome contains both essential and non-essential genes. b) Deletion of the non-essential UL43, VMW65 and ICP34.5 region results in the production of the replication competent 1764 strain.

The shuttle vector contains sequence derived from the viral locus to be targetted. In the final shuttle vector the transgenic sequence is flanked by 1 – 2 kb of viral sequence on either side such that when the plasmid containing the shuttle construct is linearized and co-transfected with the virus genome (Fig 2.1) into eukaryotic cells leading to the formation of recombinant plaques (in addition to non-recombinant plaques which only contain the parent HSV genome). It has been shown that between 0.1% and 1% of these plaques represent a new recombinant strain when the calcium phosphate transfection method is used (Burton et al., 2002).

Figure 2. 2 homologous recombination in HSV 1764 27- 4-

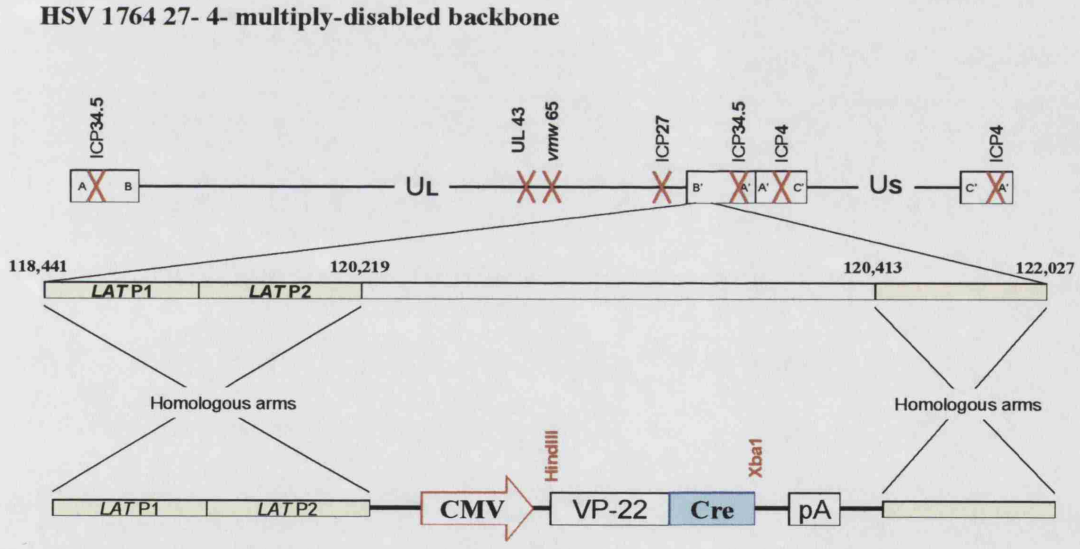


Figure 2.2: Creation of 1764 27- 4- CMV VP22-Cre virus; 1) the deletion of the two essential immediate-early genes ICP27 and ICP4 results in a replication-incompetent HSV-1 vector with an improved cytotoxicity profile. 2) Homologous recombination between the HSV flanking arms of the pR19 shuttle cassette incorporated the transgene

into the inverted repeated section bounding the U_L region. Note that two insertions events are possible one in either terminal repeat section.

By deleting essential genes from the virus genome and providing them *in trans* though cell-lines engineered to express these genes, replication deficient viruses such as 1674 27- 4- (Figure 2.2), which do not contain a full complement of essential genes, can be cultured. This method of vector development also achieves reductions in the cytotoxicity and pathogenicity of the recombinant viruses as they are replication incompetent and express fewer viral genes. By deleting immediate-early genes in this manner nearly all of the native viral proteins can be tuned off leading to much safer vector subtypes (Krisky et al., 1998).

Figure 2. 3 HSV 1764 GFP-Cre virus

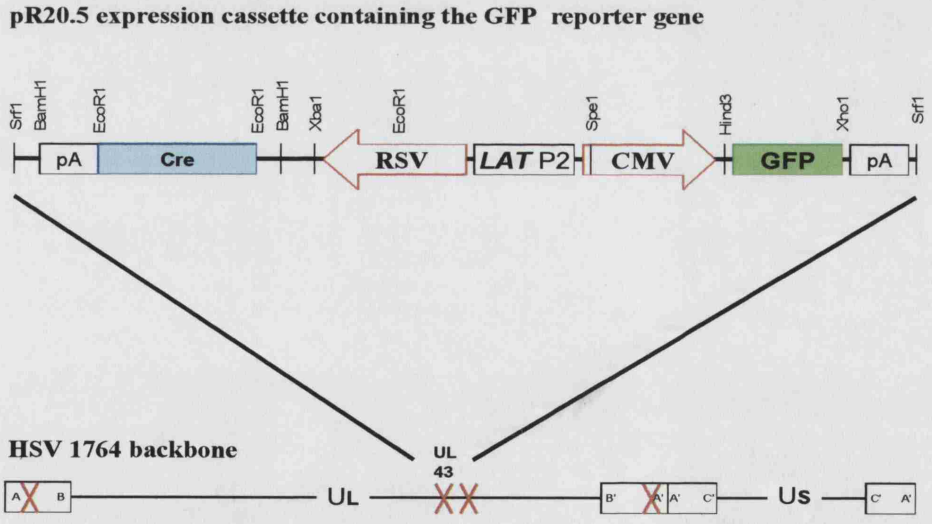


Figure 2.3; The introduction of the pR20.5 shuttle vector containing the GFP reporter gene & Cre recombinase into the UL43 region of the replication competent HSV-vector 1764.

With the introduction of reporter genes into HSV vector systems (Figure 2.3), a simple and rapid detection system for the determination of recombinants has been

achieved. These vectors allow for quick and high throughput screening of viral plaques for novel recombinants (generally demonstrated by the replacement of and therefore loss of reporter function) which are then subjected to further analysis by Southern blot techniques (Krisky et al., 1997).

2.2.2 HSV vectors to drive long-term gene expression with minimal toxicity.

2.2.2.1 Controlling the replication capabilities of the vector system

Elimination of viral replication is the first goal of any therapeutic HSV-based vector system. There are three strategies currently in use to eliminate the lytic infection and viral reproduction and improve the safety of the vectors.

The first method leads to the production of conditionally replicating vectors. These viruses have one or more non-essential genes deleted. This change results in a particle which can generally replicate *in vitro* but is unable to do so *in vivo* with few exceptions. The deletion in ICP34.5 represents one such conditional replicant. ICP34.5 (infected cell protein 34.5) is a non-essential gene which when deleted results in a virus which can replicate *in vitro* but not in quiescent cells (such as neurones) *in vivo* (Coffin et al., 1996). In cancer cells however, the lytic cycle of this virus is activated allowing its replication to occur (Rampling et al., 2000).

The second method created a replication defective virus. In these viruses one or more immediate early genes (ICP0, ICP4, ICP22, ICP47 and ICP27) are deleted and the propagation of these viruses can only be accomplished by supplying these proteins externally (usually using cell lines engineered to express them *in trans*). This method of vector construction benefits from a reduced cytotoxicity profile as the deletion of the immediate-early genes reduced the total level of viral proteins (which are cytotoxic) expressed. Without the supply of the deleted proteins these vectors are completely unable to replicate *in vitro* or *in vivo* (Fink and Glorioso, 1997).

The amplicon method of virus generation supplies the entire vial genome *in trans* resulting in vectors which contain little viral genomic material. To generate such vectors the HSV genome minus the packaging signal and eukaryotic replication signals is engineered into a bacterial artificial chromosome and co-transfected with the amplicon

which contains the transgenic sequence flanked by the packaging signal. In the eukaryotic cell all the necessary viral coat proteins, surface glycoproteins etc are manufactured from the BAC and the amplicon sequence is packaged into them resulting in a replication defective virus particle with minimal viral genome. While these vectors provide several advantages over the former two systems, difficulties with the production of high viral titres has retarded conversion to this method of vector production and further improvements to the system are needed (Suter et al., 1996).

2.2.2.2 Achieving long-term, high level gene expression

The latency loci of HSV are found within the flanking repeated region enclosing both unique regions in the genome. These loci contain genes and putative control regions LAP1/LAP2 (Latency associated promoters 1/2) which are important in the establishment, maintenance and breaking of the latency phase of the viral life cycle. The only viral genes which are expressed during latency are those encoded by the LAT (latency associated RNA transcripts) region although their function is not entirely categorised (Burton et al., 2002).

The expression of the LAT proteins during latency, probably under the control of the LAP1/LAP2 promoter region, has provided a clue to achieving transgene expression during latency. By using the *cis*-acting regulatory elements of the LAT region several transgenes have been successfully expressed during latency, including reporter genes such as GFP and β-Galactosidase, allowing the characterisation of various constructs in terms of their efficiency and expression profile (Lachmann and Efstathiou, 1997; Arthur et al., 2001; Palmer et al., 2000).

2.2.2.3 Vector targeting

The broad host range of the HSV-1 virus provides a useful starting point in the development of vectors with a more restricted expression pattern. Investigation into the endogenous promoter systems available to drive transgene expression in the native genome has provided the first generation of vectors which has shown limited success in providing both tissue-specific and temporal gene manipulation.

The use of putative LAT promoters such as LAP1/LAP2 to drive transgene expression during latency has been described above. The manipulation of alternative aspects of virus biology may provide further means of achieving targeting viral infection and/or transgene expression.

Attachment of virions to cells occurs through the interaction of viral surface glycoproteins gC and gB with glycoaminoglycan (GAG) moeities on the cell surface proteoglycans (Williams and Strauss, 1997; Laquerre et al., 1998b). The mutation gB with a lysine rich area identified as the binding domain in gB, deleted in concert with a total gC deletion, has been shown to remove nearly all viral binding to cell membrane GAG whilst retaining the viral entry functions (Laquerre et al., 1998b). This HSV mutant backbone provides a starting point in the engineering of vectors with particular proteoglycan residues displayed on their surface. The display of particular residues on the surface which match receptors on the cell to be infected may focus the binding and uptake of the vector increasing transduction efficency. These vectors are currently in development and early results have so far demonstrated proof of the principle (Laquerre et al., 1998a).

Another viral surface glycoprotein (gD) may present an alternative to the engineering of the cell attachment receptors gB/gC. The presence of unmodified gB/gC on the HSV particle facilitates its attachment to all cells displaying GAG, in contrast, the gD protein mediates entry of the virus through its linkage to a fusogenic signal present of the cell surface. If the gD protein could be modified to recognise alternative cell surface proteins which are restricted to the subpopulation of cells, control of viral entry could be achieved (Burton et al., 2002).

HSV can also be pseudotyped in a similar manner to that done with HIV-based lentiviral vectors (Naldini et al., 1996). This process involves the insertion of a surface receptor from another virus such as the G-protein of Vesicular stomatitis virus (VSV) into the vector in an attempt to alter and/or widen the host range of the virus. HSV vectors with VSV G-protein insertions have been created but did not produce vectors with the foreign glycoprotein efficiently packaged into the viral envelope. This was overcome by fusion of the transgene to the gD or gB domain. However, the viruses did not achieve efficient infection rates (Anderson et al., 2000).

Increasing the host range may not be the only route to increasing the number of cells expressing a transgene of interest. VP22, an HSV tegument protein has been demonstrated

to traffic from the cytoplasm of infected cells into neighbouring uninfected cells (Elliot and O'Hare, 1997). This protein has also been shown to effectively shuttle reporter proteins fused to it (Elliot and O'Hare, 1999). Fusion of VP22 or portions of its sequence to the C-terminal of transgenic sequences may allow the shuttling of the fused proteins from primarily infected cells to secondary cells without replication of the virus after infection.

2.2.3 Recent applications of HSV-based vectors to manipulate the PNS.

The peripheral nervous system is thought to be one of the most promising target areas for HSV-based vector systems due to the natural tropism of the virus. Viral attributes such as latency and reactivation are already targeted to these tissues and it is assumed that there exists a degree of natural optimisation in the delivery mechanisms and long term expression capabilities of HSV-based vectors within the peripheral nervous system.

Peripheral nociceptors form a subclass of sensory neurones whose cell-bodies are located in the DRG, and constitute a major target for intervention. Bipolar nociceptors, which relay nociceptive information to the spinal cord through excitation of their terminals located in the periphery, form the first link in the chain of events leading to a pain sensation. The nociceptor population of neurones have been shown to consist of cells with unmyelinated (C-fibres) as well as cells issuing thinly myelinated axons (Aδ-fibres). The majority of nociceptors have C-fibres and the cell bodies from which they issue are small diameter (<25μm) and contain peripherin which can be detected immunohistochemically. A smaller proportion of Aδ-fibre bearing cells appear to relay nociceptive information (Lawson, 2002) and can be detected immunohistochemically by the abundance of neurofilament proteins.

The characterisation of the infection profile of a possible vector system is therefore of prime importance in deciding its suitability in addressing a particular question. An efficient and reproducible infection profile by such vectors is also of importance in order to produce intelligible *in vivo* data, which remains the ultimate goal of viral gene delivery systems.

To date several applications of HSV derived vectors have been investigated in the peripheral nervous system. Trophic factors, such as NGF have been efficiently expressed in cultured neurones where they successfully protected neurones from oxidative insult (Goins et al., 1999). This type of insult is often accompanied by neuropathic pain and provides some evidence for the alleviation of such states with such a vector *in vivo.*

Neuropathic states produced by the administration of cisplatin (Aloe et al., 2000), which causes sensory polyneuropathy at doses as low as 200 mg/m^2, accompanies its use as a chemotherapy agent in the treatment of cancer (Quasthoff and Hartung, 2002). HSV-derived vectors expressing either NGF or NT-3 (Chattopadhyay et al., 2004) which have been demonstrated to be neuroprotective during cisplatin stress (Pradat et al., 2002) were successfully used to infect DRG neurones. Infection of these neurones via a subcutaneous route, prior to cisplatin administration has been shown to prevent many of the electrophysiological, histological and behavioural consequences of the induced neuropathy in rats (Chattopadhyay et al., 2004).

This group also achieved a similar neuroprotective effect during the pyridoxine overdose model of sub-acute sensory neuropathy, using HSV-derived vectors expressing NGF or NT3 in the DRG neurones (Chattopadhyay et al., 2002; Chattopadhyay et al., 2003).

The second major application for HSV vector gene-delivery in the PNS currently under investigation is in the area of chronic pain. HSV vectors carrying the pre-proenkephalin gene have been produced which produce enkephalin in both *in vitro* and *in vivo* systems (Wilson and Yeomans, 2002).

Enkephalin is the post-translational product of pre-proenkephalin, which is normally expressed in the spinal cord (Honda and Arvidsson, 1995; Comb et al., 1982). Enkephalin is stored in vesicles located in the central terminals of spinal interneurones and functions as a neurotransmitter upon its release, modulating pain transmission in the dorsal horn. Enkephalin binds to opioid receptors located on the primary afferents and second order neurones (Honda and Arvidsson, 1995). The activation of opioid receptors by enkephalin leads to the inhibition of neurotransmitter release from these neurones, reducing the firing of primary nociceptive neurones upon stimulation and also reducing sensitisation processes in primary sensory neurones (Glaum et al., 1994; Kohno et al., 1999).

The administration of a conditionally replicating HSV-pre-proenkephalin vector via footpad injections was shown to lead to the correct processing of pre-proenkephalin to enkephalin as well its storage in central terminals of sensory axons. The sensitisation of these neurones with pharmacological agents (capsaicin) was reduced or abolished in the virally treated animals compared with control groups (Wilson et al., 1999; Wilson and Yeomans, 2002). Another study using replication deficient HSV-proenkephalin (Human form) vectors demonstrated a reduction the chronic (second) phase of pain in the formalin model in rats demonstrating a possible clinical application in chronic pain management (Goss et al., 2001).

The manipulation of intracellular proteins such as ion channels in DRG neurones by HSV-based vectors has not been demonstrated so far. In contrast to neurotrophins or other extracellularly acting proteins where high expression levels are the main goal due to the their excretion into the extracellular fluid followed by their activity on the surface of neighbouring cells, any manipulation of genes encoding for intracellularly-retained proteins must be closely targeted to the subset of neurones in which the particular protein is expressed either in the naive or challenged state. It is imperative therefore that the infection profile of the vector matches closely that of the gene under investigation achieving a high degree of penetration of the target population of cells, a controlled and specific infection profile and long-term expression of the transgene from a non-virulent, non-toxic vector.

2.3 Objectives

Figure 2.4 Outline of HSV-Cre vector introduction and action.

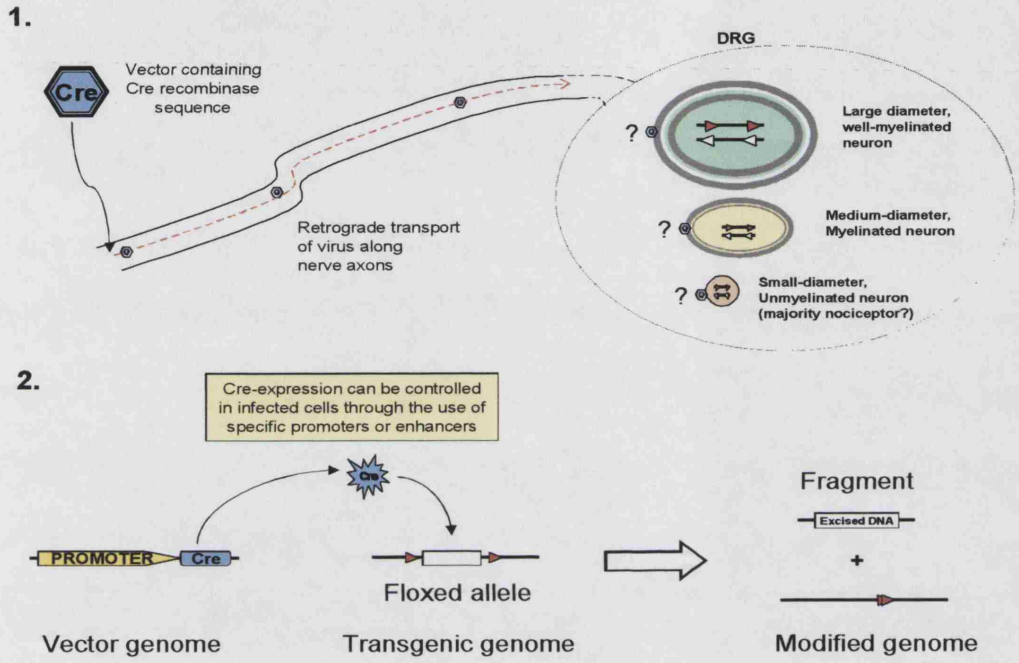


Figure 2.4: Outline of experimental design. 1.) Modified HSV vectors containing Cre-recombinase were introduced into the transgenic animal either though peripheral application or directly into the nerve. From here the virions are transported retrogradely to the DRG where they infect the cell bodies. There are three neuronal classes large, medium and small-diameter neurones. The proportion of these cells which are infected successfully is not known at present. The transgenic animal contains a target genetic sequence modified such that a defined strech of its DNA is bracketed by loxP sties (shown by red or white triangles). This target sequence can be excised though the action of Cre-recombinase. 2.) The successful translation of the viral genome containing the Cre-recombinase under the control of a chosen promoter catalyses this reaction, through the expression of Cre-protein, leading to the excision of the sequence only in transduced cells.

This study undertook to determine whether an HSV vector that expressed Cre-recombinase preferentially in defined sunclasses of neurones could be created. In particular the small-diameter neurones which constitute the majority of nociceptors were to be targeted.

In order to achieve this a series of HSV vectors, all containing the Cre-recombinase sequence and/or a GFP reporter gene were injected in adult Rosa-26 reporter (Rosa-26R) mice using various peripheral and intra-nerve application routes (Fig 2.4 [1.]). The most successful vector/introduction route was then selected for further assessment and development. Counts to establish the percentage of neurones sucessfully infected as well as co-localisation studies will be undertaken in an attempt to determine the attributes of the successfully infected DRG neurones.

A second study was done to engineer a replication deficient recombinant virus containing Cre-recombinase fused to VP22 trafficking protein in an attempt to increase Cre-expression in the neuronal target population (including small-diameter neurones). Two versions of VP22-cre cassettes were constructed, the first included the nuclear localisation signal (nls) present within the Cre sequence. The second construct was engineered to remove the nls to determine whether its presence would counter the trafficking tendencies of the fusion protein, by signalling protein retention in the endoplasmic reticulum.

The development of an efficient viral system to successfully infect and drive exogenous gene expression in adult nervous tissue, particularly in nociceptors, will allow the study of gene function in the context of the mature nervous system. The ablation of many genes early on during development has been found to lead to severe developmental defects. Many genes which are centrally implicated in pain pathways cannot be deleted due to the induction of lethal phenotypes. The use of viral vectors coupled with Cre-recombinase mediated gene deletion protocols offers the possibility of ablating these sequences in adults in a more controlled manner thus avoiding any detrimental developmental consequences.

2.4 Methods and materials

2.4.1 Molecular biology techniques

2.4.1.1 Small scale plasmid DNA preparation

The Concert plasmid purification kit was used (Gibco BRL, UK) to recover plasmid DNA from bacterial culture. Miniprep DNA harvests which deliver yields up to 20μg for an average copy number plasmid such as pBLUEscript SK were used for preparing cloned DNA constructs, DNA probe preparations and sequencing reactions. The protocol was followed as described by the manufacturers (see www.lifetech.com) and uses an alkaline/SDS procedure (Sambrook et al, 1989) to separate plasmid DNA from genomic DNA, followed by extraction of the plasmid DNA using a negatively charged anion exchange resin column. The DNA is eluted under high salt conditions, desalted, and concentrated with an alcohol precipitation step before resuspension in distilled water (20μl/miniprep).

2.4.1.2 Large scale plasmid DNA preparations

Concert High purity plasmid purification system (midi/maxi prep) (Gibco BRL, UK) which delivers yields up to 100μg of DNA per midiprep were used to prepare larger quantities of plasmid DNA for transfection protocols and sequencing.

2.4.1.3 Analytical restriction digests

Digests were done in a total volume of 10μl using 0.1 volumes of restriction enzyme and 1 x corresponding restriction buffers supplied with the enzymes without added BSA (bovine serum albumen). Reactions were incubated according to manufacturer's instructions (generally 37^{O}C for 4 hrs) then run on an agarose gel (1.5%) with ethidium bromide made up in 1 x TAE buffer for analysis. DNA fragments were visualised with UV illumination.

2.4.1.4 Restriction digests for cloning or transfection

All digests were done in a total volume of 30µl using 0.1 volumes of restriction enzyme and 1 x buffer as supplied by the manufacturer. Reactions were incubated according to manufacturers instruction for 4 hrs then run on a low melting point agarose gel (0.6%) made in 1 x TAE buffer for cloning procedures or purified with GFX PCR DNA and Gel Band Purification kit (Amersham, UK)

2.4.1.5 Blunt ending of DNA fragments

To blunt 3' overhangs of DNA, fragments were treated with 15 units of T4 DNA polymerase and 1 µl of 25mM dNTP's (dATP, dCTP, dGTP & dTTP). Reactions were left at 37^{O}C for 1 hr and purified as above.

2.4.1.6 Ligation of DNA fragments

DNA ligation reactions were performed in a standard volume of 30µl containing 3 units of T4 DNA ligase with 1 x ligase buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM ditriotheitol, 1mM ATP, 25µg/ml BSA) (New England Biolabs). DNA fragments were used in appropriate ratios and obtained directly from excised LMP gel slices (see above). The ligation reaction was left for 3 hrs at RT before transformation into competent cells.

2.4.2 Competent Cells

Competent *E. coli* cells were made by inoculating 100ml of Luria Broth (LB) with 50µl of overnight XL-1 Blue (*E. Coli* – strain) culture. The cells were grown to an optical density of 0.4 units at 580nm before they were collected by centrifugation at 1,000 rpm, RT and resuspended in 10 ml of 100mM ice cold $CaCl_2$. Cells were then re-spun as before, the supernatant removed and the pellet resuspended in 4 ml of 100mM $CaCl_2$. The cell suspension was incubated on ice for 15 min before use. Transformed cells were used within 48hrs.

2.4.2.1 Transformation of Competent cells

200µl – 1ml of competent cells were mixed with the DNA and left for 20min on ice. The mixture was then heat-shocked for 90 s at 42^{O}C, cooled on ice for 2 minutes, and incubated with shaking in LB at 37^{O}C for 1 hr. The transformed cells were then plated on to the appropriate antibiotic selective agar plates (generally ampicillin 100µg/ml) and cultured o/n at 37^{O}C.

2.4.3 Cloning of pR19VP22nlsCre

2.4.3.1 Excision of Cre sequence from plasmid

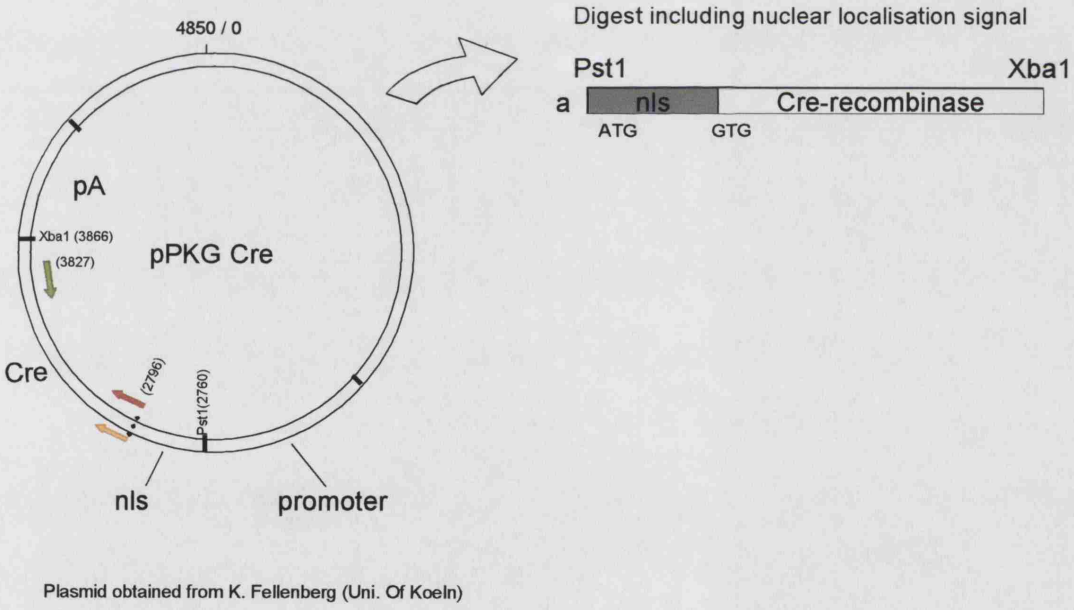


Figure 2.5 pPKG Plasmid map

The isolation of the Cre-recombinase was done by excising a 1.1 kb *Pst*1-*Xba*1 fragment from pPKG plasmid which contained all of the Cre sequence including a nuclear localisation signal at the 5' end. The *Pst*1 end was blunted with T4 polymerase with the *Xba*1 (3'-end) retaining its overhanging nucleotides.

2.4.3.2 Fusion of nls Cre sequence after VP22 sequence (pAS3)

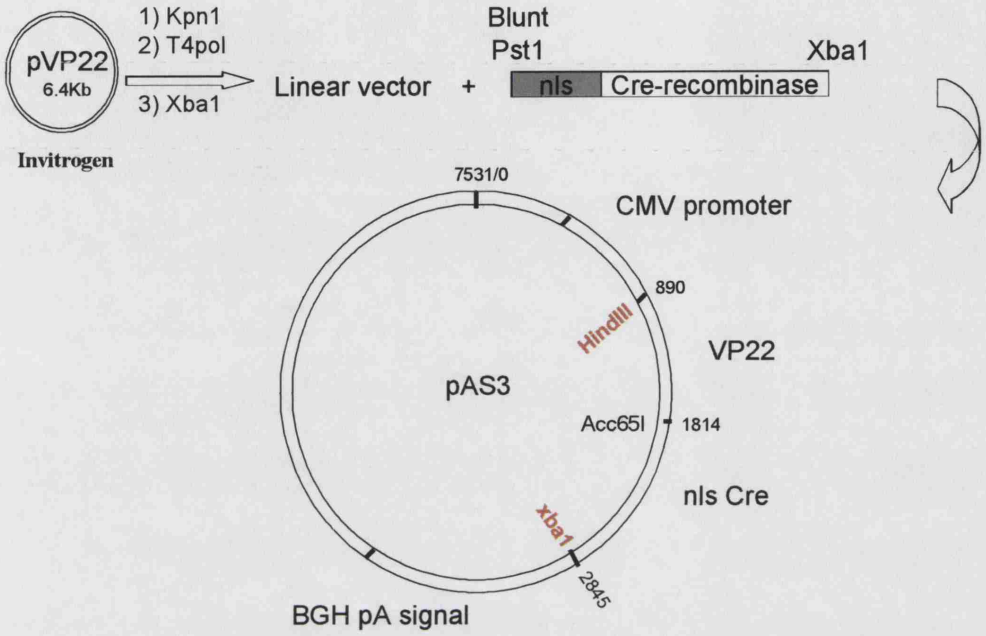


Figure 2.6 pAS3 with VP22 nlsCre sequence

The *Pst1-Xba1* fragment (1kb) obtained from pPKG was ligated into the pVP22/myc-His vector (Invitrogen) (cut with *Kpn1*/T4pol/*Xba1*) to give pAS3 plasmid containing the VP22nlsCre fusion construct (1.9kb).

The sequencing of the VP22-nls Cre construct in pAS3 was done using the T7 forward primer located immediately before the VP22 region in pAS3 plasmid (bases 863-882, Invitrogen). The sequence was checked over the fusion boundary. A series of restriction digests was also used to confirm the correct insertion of the Cre sequence.

2.4.3.3 Cloning the vp22nlsCre sequence into the pR19 shuttle vector

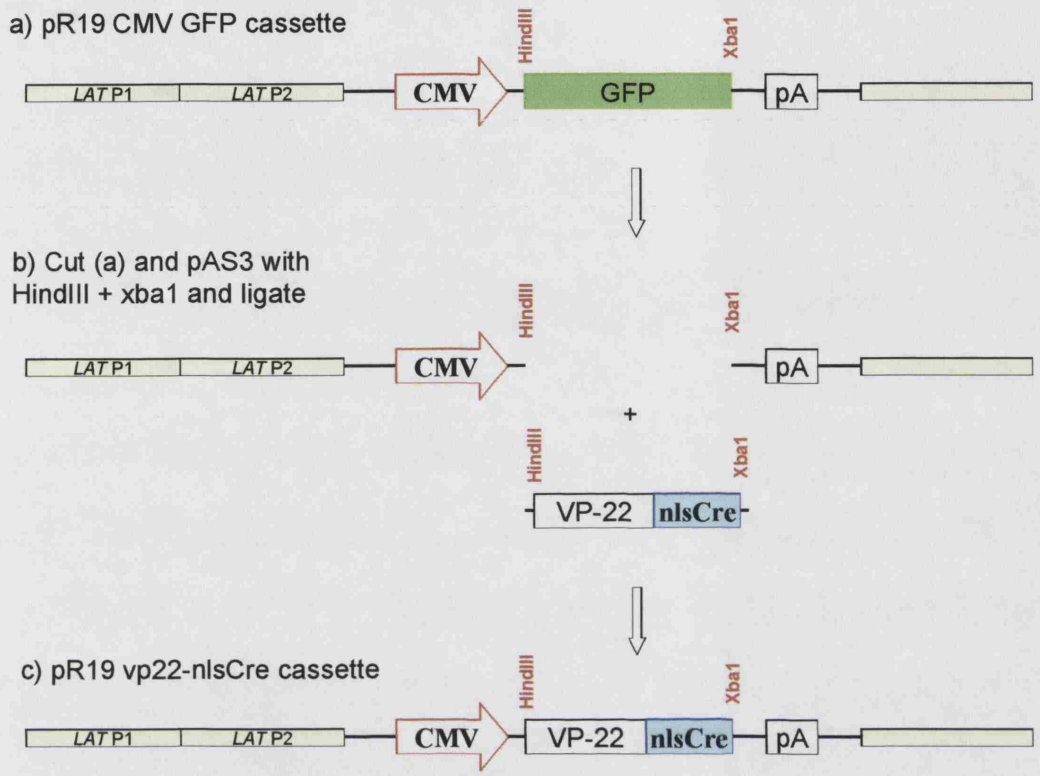


Figure 2.7 pR19VP22-nlsCre cloning strategy

Figure 2.7: cloning strategy to make pR19VP22-nlsCre shuttle vector (9.4kb) a) original pR19 shuttle vector containing function GFP (8.2KB), b) shuttle vector cut with *HindIII* and *Xba1* to release GFP sequence and VP22-nls-Cre sequence inserted, c) pR19 VP22-nlsCre shuttle cassette (9.4kb)

2.4.3.4 Sequencing of pR19 VP22-nlsCre vector

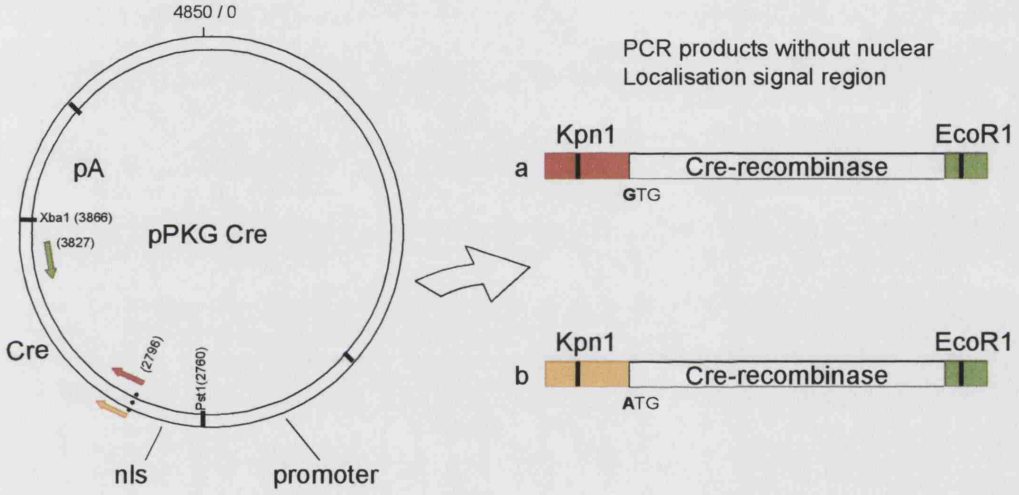
To sequence the entire VP22-nlsCre insert in the pR19 shuttle vector, two internal primers were used. The forward primer FCMV630 binds to the 3' end of the CMV promoter region, with the reverse primer sequence designed to bind to the HSV flanking region immediately following the polyadenylation signal (pA)

Forward primer FCMV630; 5' CAG TAC ATC AAT GGG CGT GGA TA 3'

Reverse primer HSVRL1; 5' TCC CCG AAA GCA TCC TGC CAC 3'

2.4.4 Cloning of pR19VP22Cre

2.4.4.1 PCR amplification of Cre sequence without nls from pPKG Cre plasmid



Plasmid obtained from K. Fellenberg (Uni. Of Koeln)

Figure 2.8 Cre sequence amplification from pPKG-Cre plasmid

In order to remove the nuclear localisation signal (nls) present at the beginning of the Cre-recombinase sequence contained in the pPKG Cre plasmid, polymerase chain reaction (PCR) primers were designed to flank the Cre open reading frame (bp 2796 – 3827) only.

This was done to determine whether the presence of the NLS signal would counteract the VP22 mediated export by retaining the protein in the endoplasmic reticulum as directed by the nls region.

Six base-pair sequences recognised by *Kpn1* and *EcoR1* were included in the forward and reverse primer design respectively to allow the fragment to be cloned further.

Additionally two versions of the forward primer was designed, the first contained the GTG start signal of the Cre sequence contained in the pPKG plasmid but the in the second primer it was replaced by ATG to restore the original bacterial Cre-sequence start methionine.

Cre Start 1a; 5' TGG TCG ***GGT ACC*** **ATG** TCC AAT TTA CTG ACC 3'

Kpn1 site Start Cre sequence

Cre Start 1b; 5' AAG AAG ***GGT ACC*** **GTG** TCC AAT TTA CTG ACC 3'

Cre End; 5' ACG CGT ***GAA TTC*** CTA ATC GCC ATC TTC CAG 3'

EcoRI End of Cre sequence

PCR protocol

Pfu polymerase was chosen to amplify the Cre sequences from the pPKG Cre plasmid due to its greater fidelity compared to Taq polymerase.

PCR reaction mix;	0.6µl	Pfu enzyme
	3.0µl	10 x Pfu Buffer
	19.4µl	dH_2O
	3.0µl	DMSO (Dimethylsulfoxide)
	1.0µl	5mM dNTP's
	1µl	0.1µg/ml p PKG Cre plasmid/ HindIII
	1µl	Start primer (1a or 1b) 0.1µg/ml
	1µl	End Primer 0.1µg/ml

PCR Cycle program;

1	x	95°C	1 min
30	x	95°C	1 min
		58°C	30 s
		74°C	2 min
1	x	74°C	10 min

The resultant products were resolved on a 1.5% agarose gel made in 1 x TAE buffer with ethidium bromide to allow DNA fragment visualization with UV-illumination and photographed.

2.4.4.2 Subcloning PCR Products into pBLUEscript

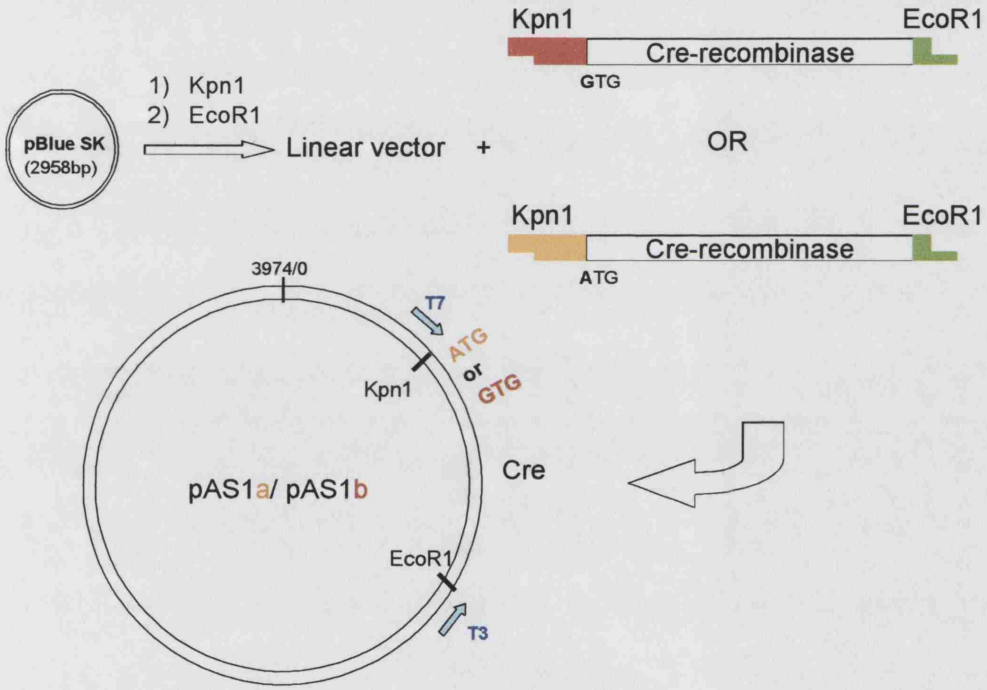


Figure 2.9 pAS1 map

The Cre minus nls PCR products obtained were sequenced by sub-cloning the fragments into pBLUEscript SK. The PCR products were digested with *Kpn1* and *EcoR1* and the double-sticky fragment (1kb) recovered from a 1.5% agarose gel. The same was done with the 2.9KB fragment obtained by double digest of pBLUEscript with *Kpn1* and *EcoR1*. These fragments were ligated with T4 ligase to give pAS1a (ATG) or pAS1b (GTG) and the resulting plasmids were sequenced using the T7 forward and T3 reverse primers present.

2.4.4.3 Fusion of the Cre sequence with VP22

The Cre-sequence from pAS1a/b was excised with *Kpn1* and *Xba1* to give the 1kb fragment with either the GTG or ATG start codon and sub-cloned into multiple cloning site of the pVP22/myc-His plasmid (Invitrogen, UK). This created the new plasmid pAS2a/b containing the VP22-Cre fusion sequence (Figure 2.10).

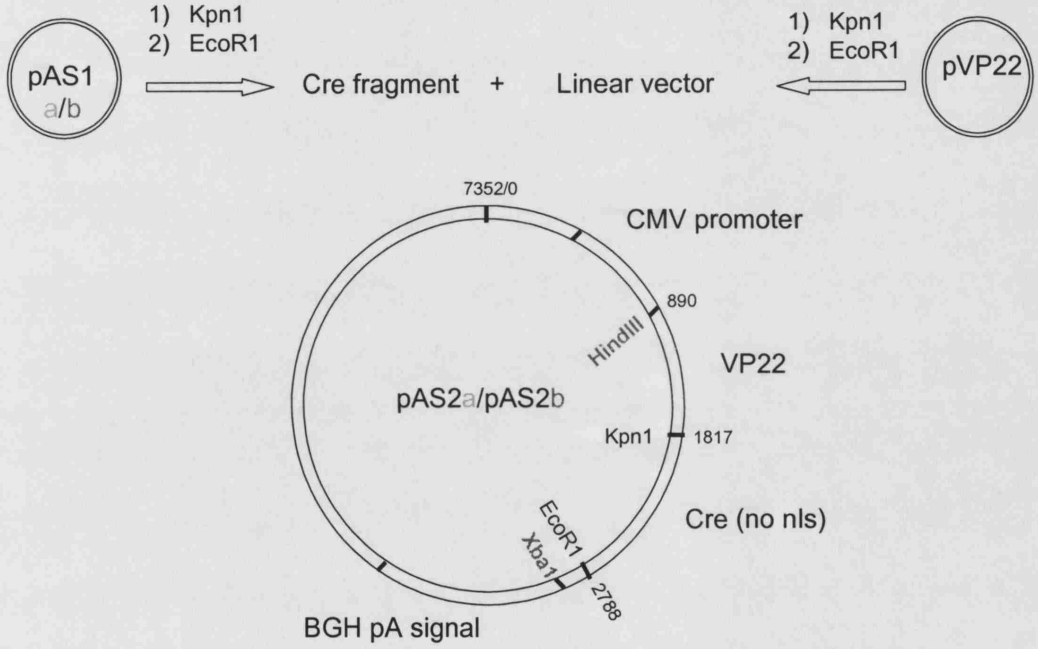


Figure 2.10 pAS2 map

Figure 2.10: Map of plasmid pAS2 containing the fusion construct between VP22 and Cre sequence (minus nls). This plasmid also contains an *Xba1* site allowing the contruct to be successfully subcloned into the viral shuttle vector in subsequent steps.

2.4.4.4 Cloning the VP22Cre sequence into the pR19 shuttle vector

The VP22-Cre fusion was cloned into the pR19 HSV shuttle vector as shown in Figure 2.11.

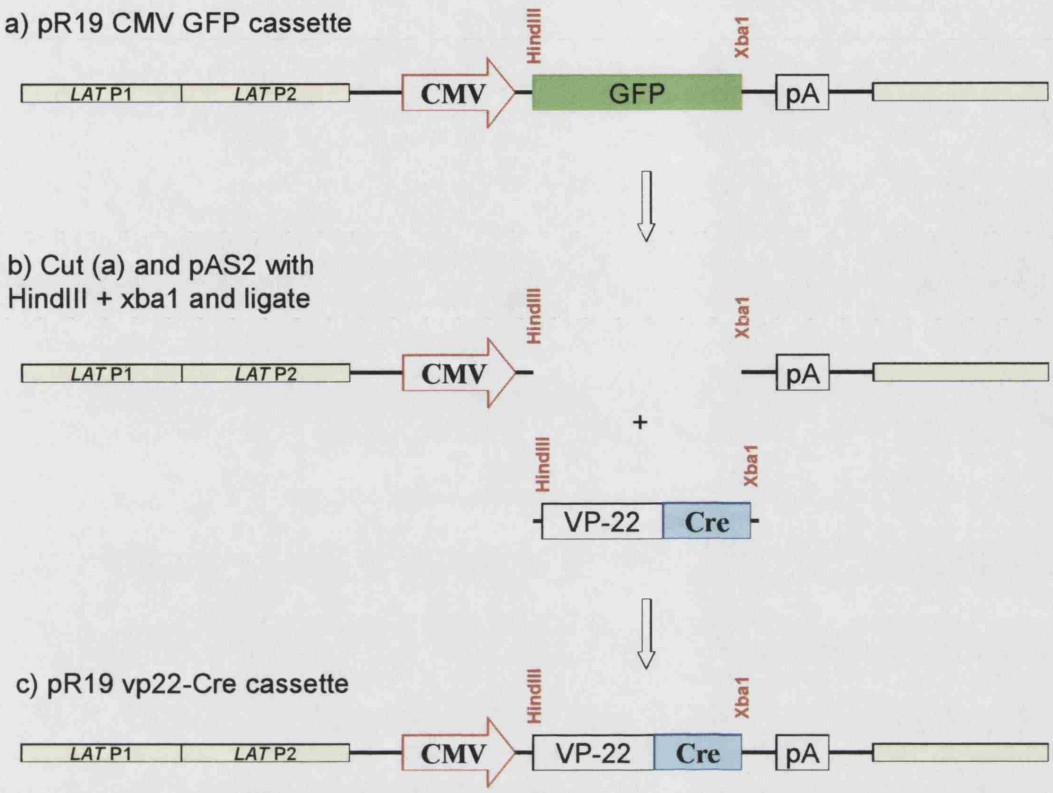


Figure 2.11 pR19VP22-Cre cloning strategy

Figure 2.11: a) the original pR19 shuttle vector containing functional GFP (8.2KB), b) shuttle vector cut with *HindIII* and *Xba1* to release GFP sequence and VP22-Cre sequence inserted, c) pR19 VP22Cre shuttle cassette (9.4kb)

2.4.4.5 Sequencing of pR19 VP22-Cre vector

To sequence the entire VP22-Cre insert in the pR19 shuttle vector, two internal primers were used. The forward primer FCMV630 binds to the 3' end of the CMV promoter region, with the reverse primer sequence designed to bind to the HSV flanking region immediately following the polyadenylation signal (pA)

Forward primer FCMV630; 5' CAG TAC ATC AAT GGG CGT GGA TA 3'
Reverse primer HSVRL1; 5' TCC CCG AAA GCA TCC TGC CAC 3'

2.4.5 Culture and passage of complimenting cell lines

Replication competent virus 1764 (Ace et al., 1989) was cultured in baby hamster kidney cells, clone 13 (BHK). The growth medium was supplemented with 3mM HMBA (Hexamethylene bis-acetamide) which is required by the 1764 HSV strain and its derivatives. HMBA is able replace VP16 (VMW65) functions in 1764 strains which contain an insertional mutation in VP16, an immediate early gene transactivator (McFarlane et al., 1992).

Replication incompetent strain 1764 27- 4- and its derivatives were cultured on BHK cells which were modified to express ICP27 and ICP4 proteins from extra-chromosomal plasmids (MaM49 cells/27/12/M;4 cells) (Thomas et al., 1999)

BHK cells were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% foetal calf serum; 5% tryptose-phosphate and 1% penicillin/streptomycin mix (sigma UK) (Full Growth Media FGMa). MaM49 cells were also grown in the above medium but were supplemented with 800μg of neomycin (G418 sulphate, Stratagene, UK) and 750μg of Zeocin (Cayla, France) per ml to positively select cells with the plasmids containing the complementing ICP27 and ICP4 sequences (FGMb).

Passage of cells of approximately 70-80% confluence was done to prevent degradation of the cell line. Cells were cultured on sterile Petri-dishes or tissue culture flasks in a humidified incubator at $37^{O}C$; 5% CO_2. Cells were detached from the flasks by treatment with 1:10 trypsin: versene solution, collected by centrifugation at 1500 rpm, 10 min, RT, and plated out at levels to allow less than <25% confluency after 24hrs. This was important as Zeocin-selectivity is operational at low cell concentrations.

2.4.6 Recombining plasmid DNA with infectious viral DNA

The engineering of recombinant viral particles takes place in the complementing cell lines. A calcium chloride transfection method (section 2.4.6.1) was used to introduce the linearized shuttle plasmid containing the genes of interest as well as the HSV viral DNA into the cells where homologous recombination occurs between sequences flanking the insertion cassette in the shuttle vector and identical sequences in the HSV genome resulting in a mix of recombinant and non-recombinant virus particles being created.

In this study the parent DNA contained the pR19GFP cassette and plaques which are formed from these particles fluoresce green when exposed to UV light. The recombinant particles do not contain GFP and result in dark plaques by contrast allowing them to be distinguished.

2.4.6.1 Transfection protocol

1) Eppendorf A containing 400µl Hebes transfection buffer (pH 7.05; RT)

140mM NaCl

5mM KCl

0.7mM Na_2HPO_4

5.5mM D-glucose

20mM Hepes

2) Eppendorf B containing DNA to be transfected

31µl 2M $CaCl_2$

1µl Herring Sperm carrier DNA (phenol-chloroform extracted)

XµL Viral DNA (1764 27- 4- pR19GFP)

Yµl Linear plasmid pR19VP22-Cre

The ratio of X: Y is variable depending on the DNA quality but is generally in the range of 8:1

3) DNA mixed and added slowly to Hebes transfection buffer (1) over 30 sec

4) 40 min at RT to bind DNA to $CaCl_2$ particles

5) Transfection mix dropped slowly on to 80% complementing cell line grown in one well of a six well plate

6) Incubated at 37 ^{O}C; 5%CO_2; 30 min

7) 1ml of FGMa added and well incubated for 7 hrs at 37 ^{O}C; 5%CO_2

8) Washed twice with 1ml FGMa (see 3.4.5 for media composition)

9) FGMa removed and cells shocked with 20% DMSO in Hebes buffer (ice-cold) for 1.5 min

10) Wells washed immediately with 2ml FGM (37 ^{O}C)

11) 2ml FGMb added (see above) and left for 3-6 days until full cytopathic effect (CPE) was observed (all cells rounded and detaching from the surface).

12) Transfection harvested and frozen (-80^{O}C) to release mature virion particles.

2.4.7 Isolation of the recombinant virus away from the parent strain

The isolation of the 1764 27- 4- pRVP22-Cre strain away from the parent 1764 27- 4- pR19GFP strain is facilitated by the fluorescent properties of the parent plaques when viewed under UV illumination. Figure 3.12 illustrates the protocol used to purify the recombinant viruses produced by homologous recombination.

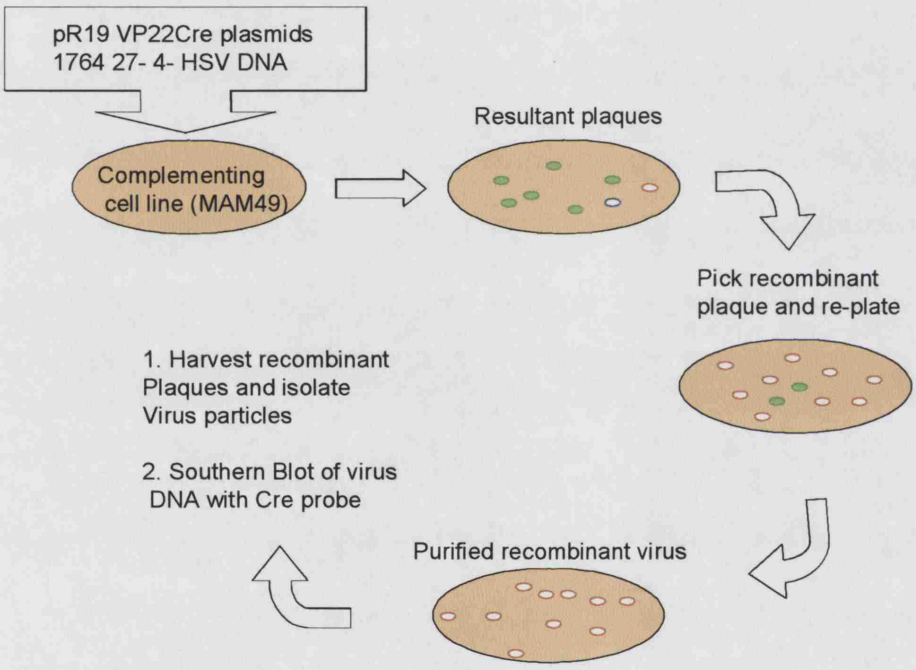


Figure 2.12 Recombinant virus production

Plaque purification protocol

1) 800 µl samples of transfection mix containing cells and recombinant virus was serially diluted in serum free media (DMEM + 5% tryptose-phosphate and 1% penicillin/streptomycin) (SFM) up to about 10^{-4} ml. Serum reduces the infection rate of HSV particles and is excluded in the infection medium.
2) A dilution series of infections was plated out at 500µl/well
3) Incubated at 37 ^{O}C; 5% CO_2; 1 hr

4) Overlaid with 2ml FGMb containing 50% CMC stock (1.6% CMC in dH_2O) to prevent virus spread away from plaques.
5) Plaques observed under the fluorescence microscope and non-fluorescent (VP22-Cre containing) plaques (recombinant) identified.10µl volume from the centre of the plaque removed with a pipette through the FGM/CMC overlay. The dilution series allowed the optimum dilution to be determined. This is one in which the plaques are well separated and recombinant plaques can be observed.
6) The 10µl volume containing recombinant virus was placed in 90µl of SFM and used to re-infect further wells and eventually purify recombinant away from parental strain.

2.4.8 Preparation of viral stocks

Pure recombinant virus harvested from one well of a six well plate was used to infect a 175cm^2 flask containing 80% confluent cells of the appropriate complementing cell-line. This infection incubated at 32^{O}C for 1764 derived viruses and 37^{O}C for 1764 27-4- derived viruses was monitored until nearly all the cells exhibited CPE. The flask was then frozen to release the virus particles and this stock used to infect a further 10 x 245cm^2 plates or 10 x roller bottles.

These infections were harvested and the cells freeze-thawed to release the viruses. The resultant mix was centrifuged at 3,500 rpm to collect the cell debris and the supernatant filtered through a 5µm (Whatman Polydisc HD, USA) and then a 0.45µm filter (Millipore, UK).

Filtered supernatant was then centrifuged at 12,000 rpm for 2 hrs at 4^{O}C to collect virion particles. The supernatant was discarded and the centrifugate resuspended in 100/150µl SFM per 250 ml original filtered supernatant.

Resuspended virus was sonicated until homogenised completely (5 x 5 sec sonicating with cooling on ice between steps). The virus stock was then titred, aliquoted and stored at -80^{O}C until use.

2.4.9 Titration of virus stocks

In order to determine the number of plaque forming units (pfu) present in a particular viral preparation a 10x dilution series of the original stock in SFM was carried out and 500µl of the dilutions plated on a number of 75% confluent plates seeded with the appropriate cell-line. After 1-5 hrs incubation at 37^{O}C (depending on the level of disability of the virus strain being titrated) the plate were overlaid with 30% CMC in FGM containing HMBA where necessary to allow plaque separation to occur and observed after 1-2 days incubation for the presence of plaques.

The number of plaques present in the lowest dilution plate with a successful infection was quantified to determine the number of units/ml. Virus stocks were freeze-thawed once after titration and any virus left used at the end of an experiment discarded to prevent reduction in transfection efficiency associated with thawing.

3.4.10 Preparation of Viral DNA (DNAzol method)

1) 1 x 175 cm^2 flask of the appropriate cell cultured to 70% confluency
2) Flask infected to give a multiplicity of infection (M.O.I.) of approximately 1 for 1764 27- 4- derived viruses and 0.05 for 1764 derived stock.
3) Medium removed when cells were rounded but still attached to the plastic.
4) 5ml DNAzol (Gibco/BRL) added to the flask to lyse the cells
5) 5ml DNAzol + Cell mixture removed to a 25ml falcon tube and an equal volume of absolute ethanol was over-layered. Contents swirled to precipitate DNA at ethanol/DNAzol interface
6) DNA removed and washed with 2 x 75% ethanol.
7) DNA pellet recovered by centrifugation (5,000 rpm, 1 min, RT)
8) DNA pellet air-dried, 10 min, RT
9) DNA resuspended in 8mM NaOH, incubate RT, 1hr.
10) DNA suspension neutralized to pH8 with 0.1M Hepes and stored at -20^{O}C.

2.4.11 Southern Blot of recombinant viral DNA

The viral DNA from several plaque purified recombinant HSV 1764 27- 4- pR19VP22-Cre virus isolates were digested with *EcoRV* and probed with a 738bp Cre probe made from pPKG Cre plasmid (*BamH1-Xba1* fragment) with Prime-it II random primer labelling kit (Stratagene, UK) and 50μC [^{32}P]dCTP. The expected band was approximately 4.5kb. Positive controls used were a) linearized pPKG Cre plasmid (*BamH1*) and b) HSV 1764 27- 4- pR19GFP genomic DNA spiked with *HindIII* linearized pPKG Cre plasmid. Negative control of HSV 1764 27- 4- pR19GFP genomic DNA was also tested.

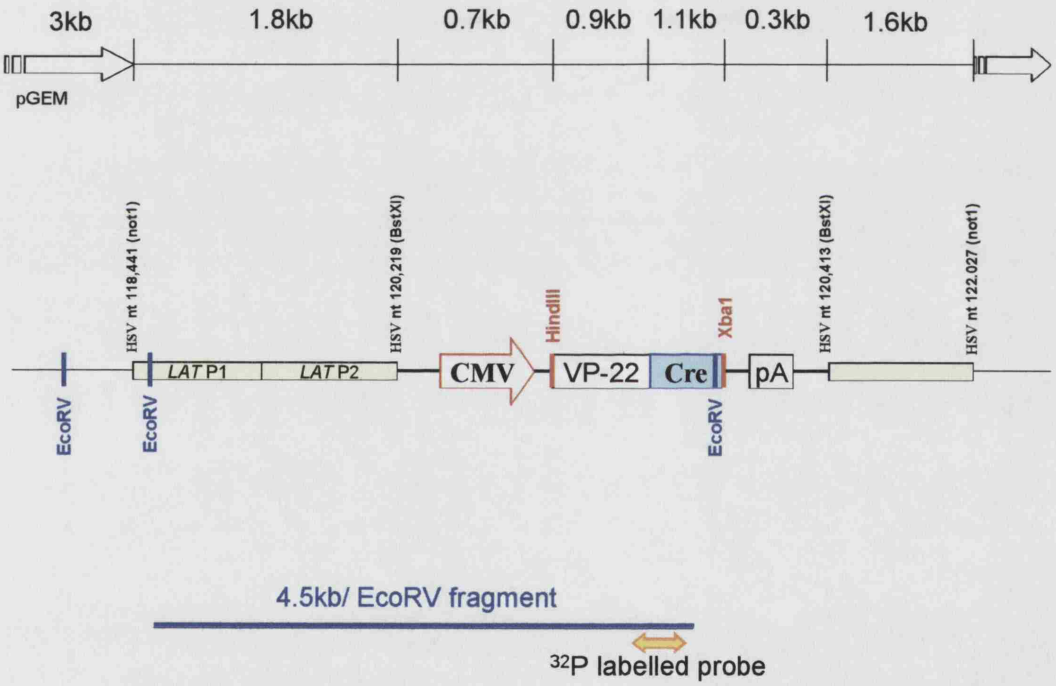


Figure 2.13 Map of pR19VP22(nls)Cre shuttle vector

2.4.11.1 Southern blot protocol

1) *BamH1/Xba1* Cre-recombinase fragment removed from pPKG Cre plasmid using low melting point agarose gel and dissolved in 3ml dH_2O per gram of gel.
2) Viral DNA samples including test recombinants restricted with *EcoRV*

3) Samples of DNA run on a 1% agarose gel (in 1 x TAE buffer until DNA fragments are resolved).
4) Illuminate gel with UV for 2 min to denature DNA in gel.
5) Denature DNA in solution A for 45min; RT with shaking

    Solution A

    i. 1.5M NaCl
    ii. 1.5N NaOH

6) Gel rinsed in dH_2O
7) 30 min in neutralizing solution B; RT with shaking

    Solution B

    i. 1M Tris pH5.5
    ii. 2M NaCl

8) Blot DNA from gel onto pre-wetted nitrocellulose membrane (Highbond N) o/n using wick method
9) Wash nitrocellulose membrane (2 X SSC)
    a. stock 20 X SSC solution C
        i. 876.6g NaCl
        ii. 441.2g Sodium citrate
        iii. 5l dH_20
10) DNA cross linked to membrane using Stratalinker (Stratagene, UK; Auto-crosslinking setting, 120,000 μJ)
11) Membrane incubated in hybridization solution; 65^{O}C; 6 hrs
    a. 1 x Hybridization solution
        i. 6 x SSC
        ii. 0.5% SDS
        iii. 5 x Denhardt's
            1. 0.5g Ficoll PM 400 (Amersham, UK)
            2. 0.5g PVP (Polyvinyl pyrrolidone)
            3. 0.5g BSA (Bovine serum albumin, fraction V, Sigma, UK)
            4. 100μg/ml denatured ssDNA (Herring sperm, Sigma, UK)
        iv. dH_20

12) Probe fragment radio-labelled with [^{32}P]dCTP using Prime-it II random primer labelling kit (Stratagene, UK)
    a. DNA solution incubated in water bath at 95^{O}C ;7 min
    b. 37^{O}C water bath; 10min
    c. Cool to RT 1 min
    d. DNA labelling bead containing klenow fragment, random hexa-oligos and buffer added
    e. 5µl (3000Ci/mmol) [^{32}P] dCTP added
    f. made up to 50µl reaction volume with dH_2O
    g. incubated at 37^{O}C; 10 min
13) Probe cleaned away from unbound nucleotides with Qiagen (UK) QIAquick nucleotide removal kit and denatured at 95^{O}C; 5 min
14) Probe cooled for 2 min; RT
15) Denatured radio-labelled probe added to hybridization tube containing membrane and hybridization solution 65^{O}C; o/n
16) Membrane washed to remove unbound probe
    a. 2 x SSC + 0.1% SDS
    b. 0.5 x SSC + 0.1% SDS
    c. 0.1 x SSC + 0.1% SDS
17) Membrane exposed to KODAK AR-Film o/n and developed to check for presence of bands.

2.4.13 Evaluation of virus tropism (*in vivo*)

The expression pattern of the HSV 1764 20.5 GFP/Cre /UL43 virus (Branston R. Gift) was investigated to determine its suitability in mediating excision of floxed genes located in the nociceptor subpopulation of DRG neurones in Rosa 26R mice. Two viral introduction routes were tested, intraplantar and intra-sciatic nerve injection routes. The assessment of cell populations expressing Cre-recombinase was done (protocol described in chapter 4) using 10µm serial sections alternatively stained for the presence of Cre activity with X-gal or for the large/small diameter subpopulations with neurofilament and

peripherin antibodies. Virus titres of between 4 x 10^8 pfu (plaque forming units)/µl and 5 x 10^9 pfu/µl were tested.

2.4.12.1 Injection of virus into the footpad

Footpad injections used 20µl of neat virus suspended in SFM introduced into the subcutaneous region of the plantar surface of the rear hind limb (right side). Rosa-26 reporter (Rosa-26R) animals (chapter 2) were anaesthetised with 5% Halothane/Oxygen mixture for the duration of the procedure.

DRG were extracted at 1 week and 1 month following treatment from both the ipsilateral (right) and contralateral (left) sides, lumbar region 1-6 and assessed for GFP and Cre-expression.

2.4.12.2 Injection of virus via the sciatic nerve

Rosa-26R mice were anaesthetised with 5% Halothane/Oxygen mixture for the duration of the procedure. The sciatic nerve was then exposed at mid-thigh level and a Hamilton syringe connected to a fine-bore glass micropipette used to inject between 2 – 5 µl of neat virus directly into the nerve. The injection of virus suspension was done over a period of 5 minutes to allow the maximal uptake of liquid by the nerve. The nerve was then recovered with the superficial tissues and the wound closed. Animals were allowed to recover and were sacrificed at 1 week and 1 month post-inoculation as before.

2.5 Results

2.5.1 Construction of 1764 27- 4- pR19VP22nlsCre virus

The cloning procedure, as outlined in the materials and methods section, was followed and lead to the incorporation of the VP22 nls Cre fusion sequence into the pR19 plasmid. The steps leading to this result are illustrated in figures 2.14 and Figure 2.18. Figure 2.14.a shows the plasmid pAS3 derived from pVP22 containing the nls-Cre sequence from pPKG Cre plasmid successfully fused in frame with the 3' terminal of the VP22 sequence. A restriction digest (Figure 2.14.b) is also shown illustrating the correct sequence of fragments achieved with a variety of enzymes. This was done to determine if the nls-Cre fragment had ligated in the correct orientation into the VP22 parent vector.

The pAS3 plasmid was sequenced using primers indicated and the inserted sequence was found to be correct in comparison to the published sequences for the VP22 gene (Invitrogen) and the nls-Cre sequence which consists of the NLS signal from SV40 large T antigen (personal correspondence K. Fellenberg nucleotides 2778 – 2798 of pPKG Cre plasmid sequence) fused to the Cre-sequence amplified from wildtype P1 phage (Accession number X03453 Genbank). The fusion was also found to be in-frame.

The successful recombination of the shuttle vector containing the pR19VP22nlsCre cassette with homologous flanking sequences replacing the GFP gene in the parent 1764 27- 4- pR19GFP virus was accomplished using a ratio of 1:10 of linearized pR19 shuttle vector to viral DNA. Very few white recombinants were found however. Twelve white plaques were isolated from various transfections and clean stocks grown on MaM49 cells. DNA prepared from these isolates was probed for the presence of Cre by means of Southern blots. None of the recombinant viruses exhibited a positive band for the Cre-sequence however (Figure 2.19).

2.5.2 Construction of 1764 27- 4- pR19VP22nlsCre virus

The construction of 1764 27- 4- pR19VP22Cre virus without the nuclear localisation signal was accomplished in a series of stages.

2.5.2.1 Isolation of the Cre sequence, without the SV40 nuclear localisation signal (nls), from the pPKG Cre plasmid.

The amplification of the Cre sequence away from the nuclear localisation signal which preceded it in the pPKG Cre plasmid was not possible using restriction digests owing to a lack of suitable unique sites. Therefore, a PCR based approach was used to amplify the Cre sequence (Figure 2.15) and also to engineer unique restriction sites on either side of the sequence.

The use of PCR to isolate the Cre-sequence also allowed the reconstitution of the start ATG signal of Cre which had been replaced with a GTG codon when the original Cre-sequence was fused to the nuclear localisation signal of SV40 to make the pPKG Cre plasmid.

Two alternative start primers using either the original GTG sequence or the new ATG sequence were used allowing the production of two forms of each of the following plasmids and viruses.

The resulting Cre-fragments with either a start ATG or GTG codon were subcloned into the multiple cloning site of pBLUEscript to allow the full sequence to be obtained using the T7 and T3 primer sites present in this plasmid. The resulting plasmids pAS1a and pAS1b are shown in Figure 2.16. The sequence was checked against the Cre-sequence from the bacteriophage P1 published in Genbank (accession number X03453) and found to be correct.

These Cre fragments were then fused in-frame with VP22 to create two plasmids pAS2a/b (Figure 2.17). The VP22-Cre insert were verified by means of restriction digests.

The VP22-Cre sequence was subcloned into the pR19 shuttle vector using the *HindIII-Xba1* fragment (Figure 2.18) and the full sequence checked by sequencing using primers located in the promoter region preceding the insertion site and reverse primers located in the HSV homologous flanking arm following the polyadenylation signal region. The sequence was found to be correct and in-frame.

The recombination of the pR19VP22Cre cassette into the HSV backbone proved difficult and only eight white plaques were isolated. These recombinants were isolated away from the parent 1764 27- 4- pR19GFP virus and grown on MaM49 cells. These viruses grew well at similar conditions to the parent strain but when Southern blot analysis

to validate the presence of the Cre-sequence in the viral DNA was carried out, none of the isolates were found to be positive (Figure 2.19).

2.5.3 The expression pattern of 1764 pR20.5GFPCre/UL43 virus in DRG

The expression pattern of 1764 pR20.5GFP-Cre inserted into UL43 (Gift Branston R.) was assessed to determine the Cre-mediated excision events achievable in DRG neurones with this virus when administered *in vivo*.

By injecting 20µl of virus suspension at titres up to 10^8 pfu/ml into the footpad it was determined that only low levels of Cre-mediated excision in L4/5 DRG ipsilateral to the injection site could be observed. The level increased slightly after 1 month p.i. but this route was unsuitable for gene manipulation in DRG *in vivo* (Figure 2.20). No GFP or Cre-mediated expression was observed in the contralateral DRG (data not shown).

Delivery of this virus via a sciatic nerve injection route was found to be more successful. Figure 2.21 shows the expression levels achieved in whole L4/5 ipsilateral to the injection site at 1 week and 1 month p.i. Titres of the virus between 1 x 10^8 pfu/ml and 7 x 10^8 pfu/ml range (5µl/ injection) were tested. As before no cells of the contralateral side showed any signs of infection (data not shown).

In order to determine which of the subpopulation of DRG cells were being infected by the virus when it was introduced via the sciatic injection route, 10µm sections were taken 1 week and 1 month after infection and counterstained with an neurofilament recognising antibody (N52) which is a marker for large-diameter, myelinated-axon bearing neurones. Figure 2.22 shows a section taken one week p.i. with 1764 pR20.5 GFPCre/UL43 (Branston R.) via the sciatic route. This section shows that a number of cells are expressing GFP and they are mainly are N52-positive cells(Figure 2.21). As can be seen from the representative pictures shown in figure 2.21 the actual transfection level achieved is quite low and is maximal around one month post-injection.

A count was done (n=6) to determine the maximal expression levels which could be achieved with this virus. The animals were left for 1 month and then 10µm serial sections taken and alternate slides stained with X-Gal to determine Cre-expression (Rosa-26R animals) or anti-peripherin (red) or anti-neurofilament (green) (Figure 2.23). Co-labelling with β-gal antibody was not performed due to technical limitations; therefore serial

sections were used to quantify the β-gal expression levels in the two subpopulations of neurones (chapter 4 methods describes protocol followed in full).

Counts were done of all positive neurones and then these neurones were sorted according to their antibody profile into small diameter peripherin-expressing neurones (mainly nociceptors) and large-diameter, neurofilament rich neurones (mainly non-nociceptors). This data is presented in table 2.1 below and summarised in Figure 2.24.

Table 2. 1 Quantification of infected neurones

Slide	Peri	N52	Total Neurones	Slide	Peri/Cre	N52/Cre	Infected neurones
1'	152	44	196	1	7	9	16
2'	190	56	246	2	6	15	21
3'	140	38	178	3	6	13	19
4'	176	48	224	4	3	8	11
5'	161	48	209	5	2	11	13
6'	150	58	208	6	9	11	20
Mean	**161.5**	**48.7**	**210.2**		**5.5**	**11.2**	**16.7**
S.E.	7.5	3.0	9.5		1.1	1.0	1.6

Table 2.1: Counts obtained from six pairs of serial sections (one stained for Cre-expression with X-gal and the other comparison slide colabelled for peripherin and N52 presence) taken from three ipsilateral lumbar 4/5 DRG one month p.i. with 5μl 1764 pR20.5 GFP-Cre/UL43 virus suspension via the Sciatic nerve route (Peri - peripherin positive neurones, N52 – N52 positive neurones, Peri/Cre – Peripherin positive neurones which also express Cre, N52/Cre – N52 positive neurones which express Cre).

Figure 2.14 pAS3 restriction analysis

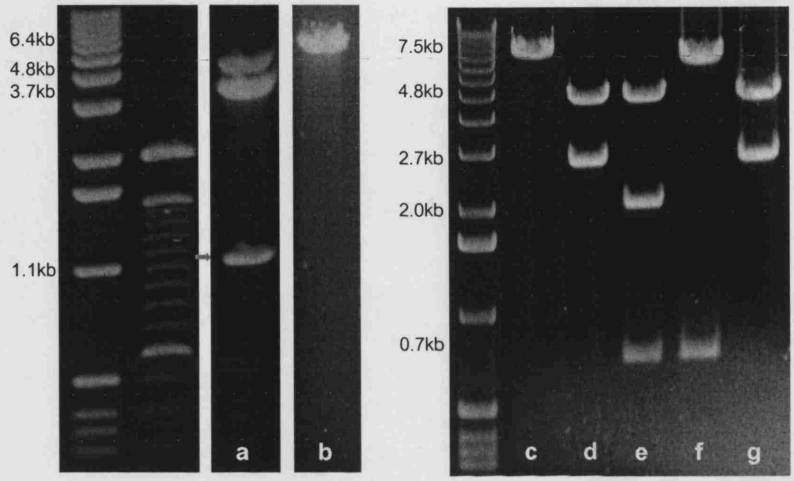


Figure 2.13 pAS3 cloning: a) pPKG Cre plasmid digested with *Pst1*; T4-polymerase; *Xba1* to release the Cre sequence including the nuclear localisation signal (nls) results in three bands 1) 4.8kb uncut plasmid 2) fragment 3.7kb 3) Cre – 1.1kb(red arrow); b) pVP22 (Invitrogen) cut with *Kpn1*;T4-polymerase;*Xba1* releases the pVP22 backbone (6.4kb) ready to receive the semi-blunted *Pst1-Xba1* nls-Cre fragment from (a), Diagnostic restriction digests [c – g] of resulting ligated vector pAS3 to check orientation of the nls-Cre insert c) linearized with *BamH1*, d) *Spe1* fragments, e) *BamH1* & *Spe1*, f) *Xba1* & *BamH1*, g) *Xba1* & *Spe1* (refer map of pAS3, Figure 2.6)

Figure 2.15 Cre fragment without nuclear localisation signal

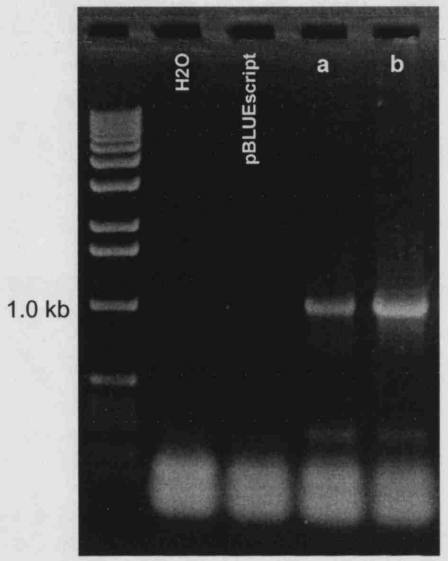


Figure 2.15: Amplification of Cre sequence from pPGK Cre plasmid to exclude the nuclear localisation signal. Cre sequence is indicated by a 1kb PCR product shown in lanes a and b but not from PCR reaction using water or pBLUEscript plasmid demonstrating specificity of primer binding; a) Cre-amplification product obtained using 1µl (0.1µg/ml) pPKGCre + Cre start 1a (includes ATG start codon), b) Cre-amplification product obtained using 1µl (0.1µg/ml) pPKGCre + Cre start 1b (includes GTG start codon).

Figure 2.16 pAS1 restriction analysis

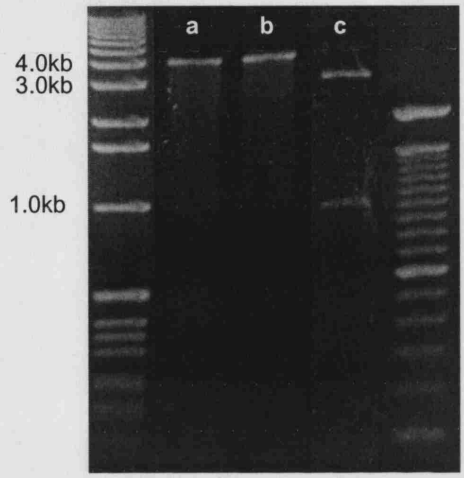


Figure 2.16: restriction digests to check the correct insertion of the Cre sequence excluding the nls obtained by PCR into pBLUEscript vector backbone (refer map of pAS1, Figure 2.9); a) pAS1 linearized with *Kpn1*, b) pAS1 linearized with *EcoR1*, c) pAS1 *Kpn1/EcoR1* digest showing 1.0kb Cre insert and 3.0kb vector backbone.

Figure 2.17 pAS2 restriction analysis

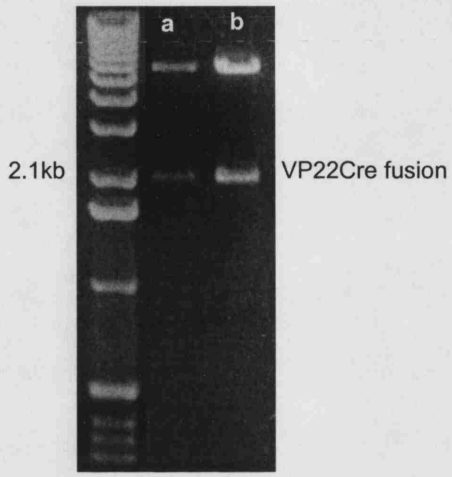


Figure 2.17: pVP22Cre (pAS2) construct restricted to show VP22Cre insert (no nuclear localisation signal, refer to map of pAS2 Figure 2.10); a) pAS2a (pVP22-**GTG-**Cre) cut with *HindIII/ Xba1* to release fusion sequence, b) pAS2b (pvp22-**ATG-**Cre) cut with *HindIII/ Xba1*.

Figure 2.18 Restriction analysis of pR19 cassettes

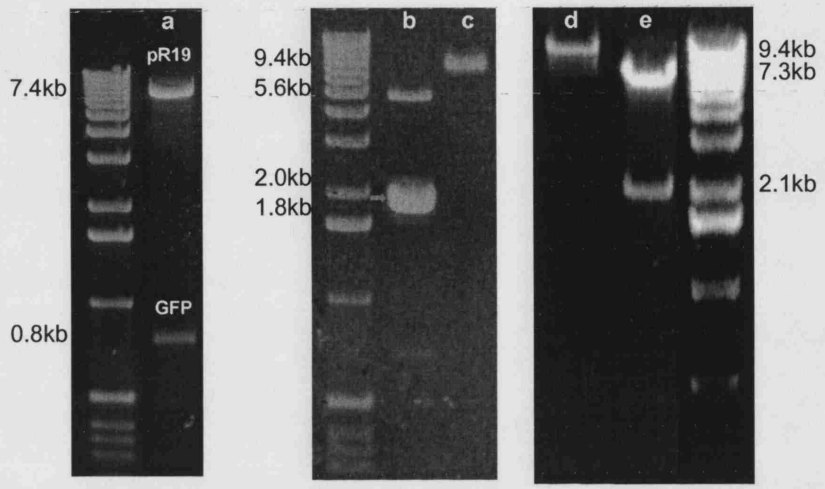


Figure 2.18: a) pR19 CMVGFP cut with *HindIII* & *Xba1* releasing GFP sequence to allow the subcloning of new VP22-Cre fusion sequences into backbone (refer Figure 2.11), b) pR19VP22-nls-cre cut with *Spe1* & *BamH1* demonstrating diagnostic restriction pattern with 3 bands (1.8kb, 2.0kb & 5.6kb, red arrow shows two bands), c) pR19VP22-nls-cre plasmid linearized with *Sca1* before transfection to allow maximal homologous recombination to occur in the complementing cell line. d) linear pr19VP22cre/*Sca1*, e) pR19VP22Cre/*HindIII & Xba1* to release VP22Cre fusion sequence insert of the correct size (2.2kb).

Figure 2.19 Southern blot analysis of recombinant viruses

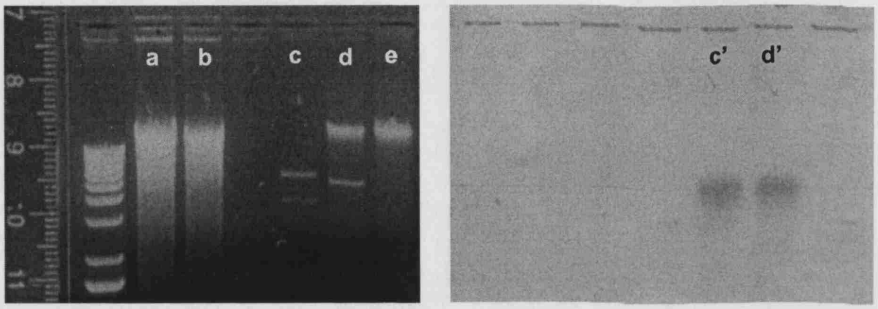


Figure 2.19: Southern blot analysis of two samples of genomic DNA harvested from putative recombinant VP22Cre white viruses. The agarose gel on the left shows the DNA samples as follows; a) 1764 27- 4- pR19VP22nlsCre genomic DNA digested with *EcoRV*, b) 1764 27- 4- pR19vp22Cre (ATG) genomic DNA/ *EcoRV* digest, c) pPKG Cre plasmid positive control d) linearized 1764 27-4- pR19GFP DNA spiked with pPKG-Cre plasmid (positive control) e) linear 1764 27- 4- pR19GFP (negative control). The right picture shows a radiogram obtained by probing DNA blots from the gel with a radioactive probe designed to hybridize to Cre-sequences; c') positive Cre band d') positive Cre band. Note that no Cre is detected in either of the two recombinant viruses tested.

Figure 2.20 Expression of GFP/Cre after infection of Rosa-26R mice with 20µl HSV1764 pR20.5GFP/Cre/UL43 (10^8pfu/ml virus suspension) via the footpad injection route (1 week and 1 month p.i.).

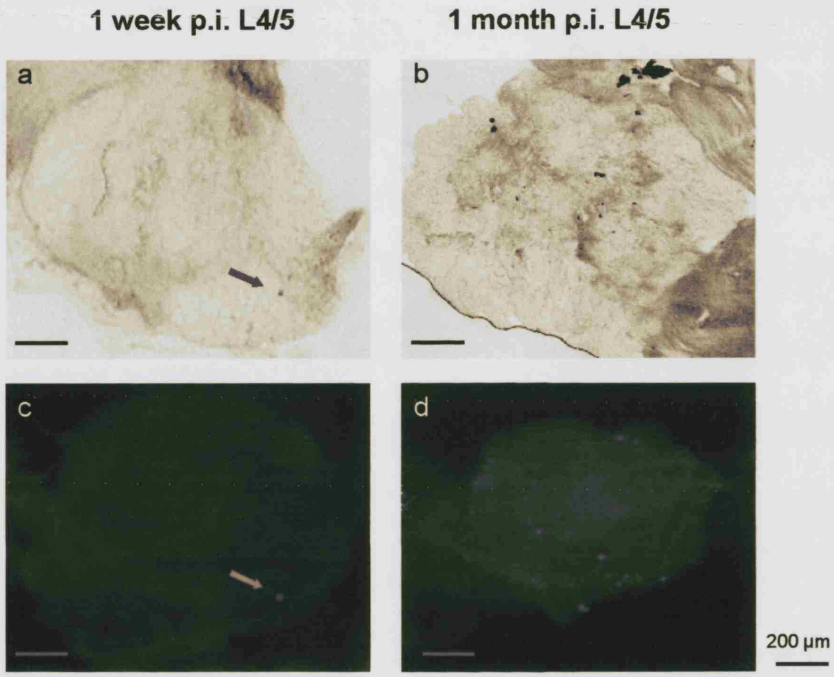


Figure 2.20: Infection rates achieved in whole L4/5 DRG with 20µl (10^8 pfu/ml) 1764 pR20.5 GFP/Cre/UL43; a) Cre-expression (as shown by a posistive X-gal test) 1 week p.i, b) Cre-expression 1 month p.i., c) GFP expression 1 week p.i., d) GFP expression 1 month p.i.

Figure 2.21 Expression of GFP/Cre after infection of Rosa-26R mice with 5µl HSV1764 pR20.5GFPCre/UL43 (10^8pfu/ml virus suspension) via the sciatic nerve injection route (1 week and 1 month p.i.).

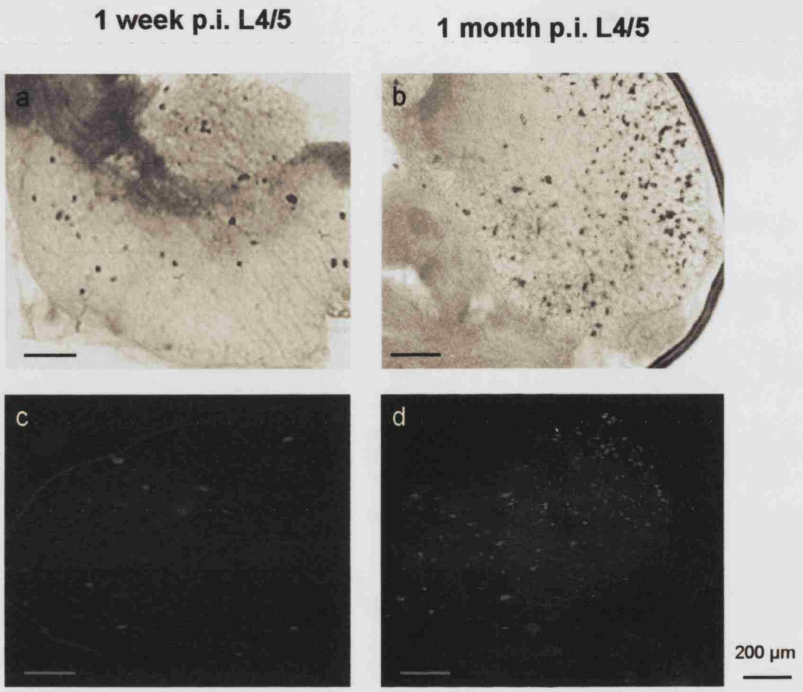


Figure 2.21: Expression pattern of 1764 pR20.5GFPCre/UL43 in whole L4/5 DRG following sciatic nerve injection of 5µl of virus suspension (10^8pfu/ml); a) Cre-expression (exemplified by blue x-gal positive cells) 1 week p.i, b) Cre-expression 1 month p.i., c) GFP expression 1 week p.i., d) GFP expression 1 month p.i.

Figure 2.22 The correlation of the GFP expression achieved in Rosa-26R mice infected via sciatic injection with 5µl HSV1764 pR20.5GFPCre/UL43 (10^8pfu/ml virus suspension) with large-diameter N52-positive DRG neurones.

Figure 2.22: GFP expression directed by 1764 pR20.6GFP-Cre/UL43 virus 1 week post sciatic nerve injection of 5µl (10^8 pfu/ml) virus suspension; this 10µm section has been counterstained with an α-neurofilament antibody (N52) which recognises medium-large diameter neurones possessing myelinated fibres. Yellow arrows indicate large cells also expressing GFP. Other cells (N52-negative) expressing GFP are shown in Green.

Figure 2.23 X-gal expression (functional Cre) in DRG from Rosa-26R mice infected via the sciatic nerve with 5µl HSV1764 pR20.5GFPCre/UL43 (10^8pfu/ml virus suspension) correlated with peripherin and N52 expression.

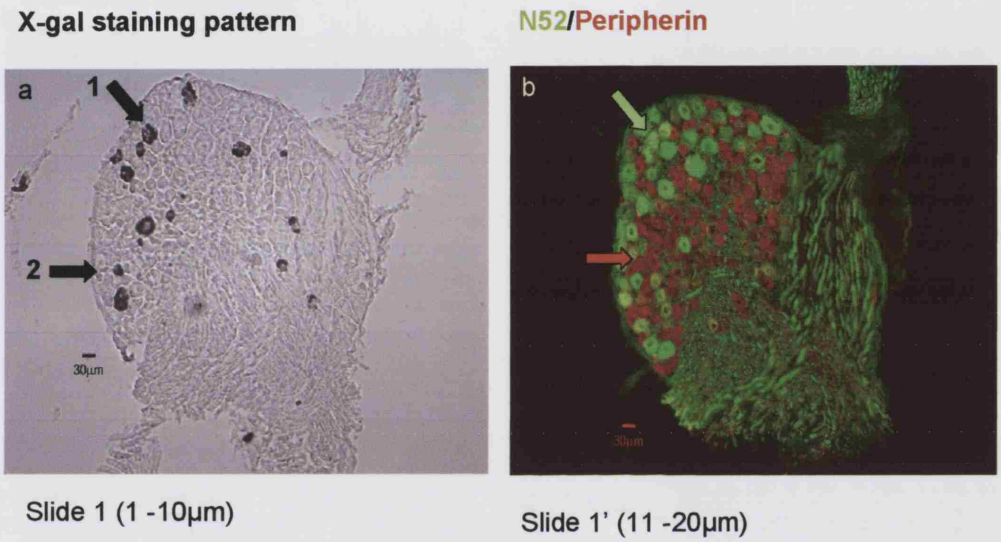


Figure 2.23: Comparison between 10µm serial sections of a DRG taken from the ipsilateral L4/5 DRG 1 month after injection of 5µl of 1764 pR20.5GFP-Cre/UL43 virus into the Sciatic nerve. The expression pattern of the Cre is demonstrated in slide1 (panel a) by the X-Gal stained cells (dark). These cells were classified into large diameter neuron expressing neurofilament (green cells panel b) or small diameter, peripherin positive neurones (red cells panel b) by comparison with the next slide in the series, slide 1' (panel b). A large diameter cell infected with the virus and expressing the pR20.5GFP-Cre cassette is indicated by arrow 1 and a small diameter one by arrow 2. The GFP expression from the virus was quenched by drying the slides for 2 hours at RT (data not shown).

Figure 2.24 maximal expression level achieved via sciatic injection of 5µl HSV1764 pR20.5GFPCre/UL43 (10^8pfu/ml virus suspension) in Rosa-26R mice.

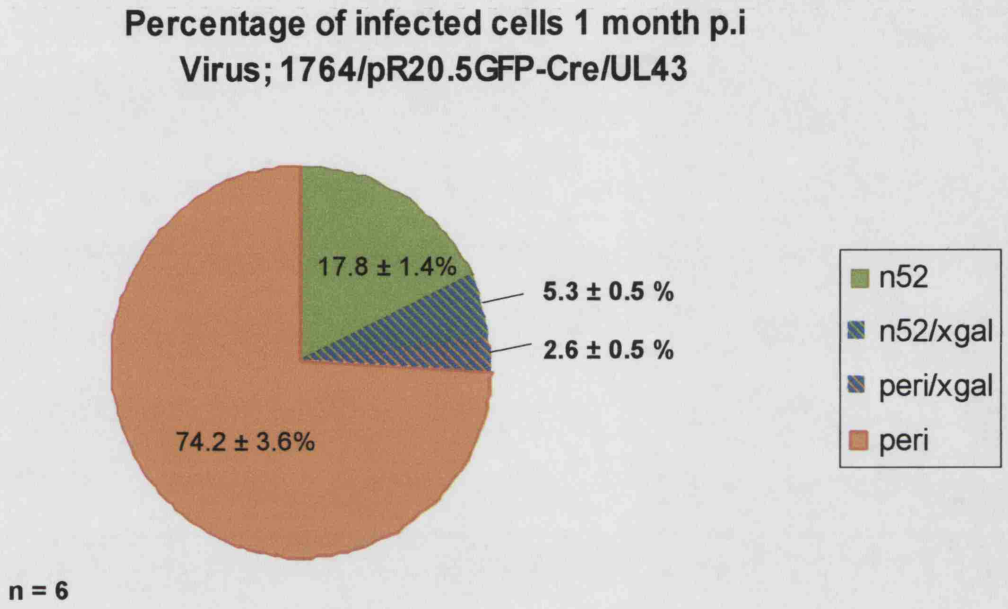


Figure 2.24: Chart showing expression levels achieved through sciatic nerve injection of 1764 pR20.5GFP-Cre/UL43 virus in Rosa-26R mice neurones of L4/5 DRG ipsilateral to the injection site; 7.9 ± 1.3% of all neurones expressed Cre one month p.i. with only 3.4 ± 0.8% of peripherin positive neurones and 23.0 ± 3.1% of N52-positive myelinated neurones successfully infected and expressing Cre-Recombinase from the pR20.5 cassette (as shown by the presence of functional β-galactosidase activity).

2.6 Discussion

In order to successfully study and modulate the initial nociceptive impulse generation by peripheral sensory neurones, methods to target these first-order neurones are required. The natural tropism of the HSV virus for nervous tissue has been exploited to achieve this by a number of groups (Hojeberg et al., 1991; Smith et al., 1995; Coffin et al., 1996; Glorioso and Fink, 2002; Dobson et al., 1990).

However, whilst a number of vectors have been developed which utilise the retrograde axonal transport capabilities of the HSV particle, to allow successful infection of DRG via a number of introduction routes (Henken and Martin, 1991; Palmer et al., 2000), no studies have yet been done to quantify the number of neurones transduced by these methods, nor which subpopulation of DRG cells are successfully infected by these vectors.

The discrete roles of sub-populations of neurones making up the DRG, particularly with regard to nociceptive information relay has become more widely understood in recent years. It is important therefore to match the infective profile of the vector system to the target cell population (expressing a gene of interest).

The central role that sodium channel $Na_v1.8$ is thought to play during inflammation (Tanaka et al., 1998; Gold et al., 1996; Lai et al., 2000; Akopian et al., 1999) makes this gene a possible target for virally-mediated modulation of the inflammatory process, allowing temporally and spatially restricted gene manipulation to be effected. The development of vectors which achieve high infection rates specifically targeted to the $Na_v1.8$ expressing cells is one example of the potential use of HSV vectors in the PNS. $Na_v1.8$ is restricted to nociceptors nearly all of which are peripherin-positive small-diameter cells. The assessment of a putative PNS infecting virus (1764 20.5 GFP-Cre) (gift Branston R.) was therefore investigated to determine its suitability in manipulating genes in such a subpopulation of neurones cells. This viral backbone (HSV 1764 transgene inserted into position UL43) had been previously demonstrated to be the most effective virus to drive transgene expression in the PNS amongst a series of vectors engineered to test various LAT promoter regions in concert with different insertion sites in the HSV genome (Palmer et al., 2000).

The data presented in this chapter shows that this HSV-1 vector, 1764 20.5 GFP-Cre/UL43 expressing GFP and Cre-recombinase (Lilley and Coffin, 2003) which has been shown to successfully infect DRG after peripheral introduction via the both the footpad and sciatic nerve routes, does not achieve infection levels above 25% in any subpopulation of sensory neurones. It also trasduces a maximum of less than 5% of the small diameter, peripherin positive neurones, the majority of which are nociceptors and the target population (Figure 2.24).

It was also determined that this maximal level of expression was achieved 1 month following inoculation into the sciatic nerve (Figure 2.21). Inoculation of the footpad (Figure 2.20) resulted in much lower infection rates than via the sciatic nerve route and therefore is not an effective introduction route for the application of these viruses although it benefits from being a less invasive method in comparison to the sciatic injection route.

The small-diameter sub-population of neurones found within the DRG is generally thought of as the most involved in the generation and maintenance of many forms of nociceptive input during both acute and chronic pain states. This vector is therefore unsuitable for the study of these conditions and/or development of pain management strategies as very few of these cells are infected successfully (Table 2.1).

In order to try and improve the infection profile of HSV derived vectors, a fusion protein of VP22 and Cre was created. VP22 is a tegument protein of HSV which has been used to successfully transport fused proteins from primarily infected cells to neighbouring uninfected cells both *in vivo* (Hung et al., 2001; Kim et al., 2004) and *in vitro* (Normand et al., 2001). This intercellular transport activity has also been noted in VP22 analogues isolated from other α-herpesviruses (Dorange et al., 2000; Harms et al., 2000).

The construction of an HSV1764 27- 4- replication-deficient virus containing VP22 fused to Cre-recombinase under the control of a CMV promoter proved problematic and none of the recombinant viruses isolated contained a functional fusion protein as demonstrated by Southern-blot analysis (Figure 2.20). In total ten recombinant white viruses per shuttle vector were isolated, grown and tested for the presence of the functional Cre enzyme. None of these viruses contained the Cre-sequence indicating that they probable represented revertants or other mutations in the GFP gene of the parental virus strain.

The failure of the correct homologous recombination event required to incorporate the VP22-Cre fusion sequence into the inverted repeating arms of the 1764 27- 4- backbone, may be attributable to the presence of approximately 1kb of HSV genome (the VP22 gene) in the transgene allowing recombination to occur between it and the native tegument protein in position UL49. This may have lead to large scale deletion of HSV genes enclosed within such a homologous event, resulting in an unstable vector, which failed to successfully infect cells.

Chapter 3; Antisense regulation of sensory neuron specific sodium channel, $Na_v1.8$ activity and localization by modulation of the annexin II light chain protein p11.

3.1 Synopsis

In vitro work has shown that the annexin II light chain molecule (p11) can regulate the trafficking of sensory neuron specific sodium channel, $Na_V1.8$ protein to the membrane of dorsal root ganglia cells. The $Na_V1.8$ channel is expressed exclusively in sensory neurones and appears to play an important role in pain pathways particularly in inflammation. $Na_V1.8$ has proved difficult to express in cell lines even in the presence of identified sodium channel accessory subunits. P11 was recently shown to increase the functional $Na_V1.8$ channel expression in the cell membrane, together with associated increases in sodium channel current densities. The possibility that p11 may be a key cofactor *in vivo* regulating $Na_V1.8$ channels remains to be clarified. This chapter provides an introduction to the annexin/p11 family of proteins, the interaction of p11 with proteins involved in nociception and pain pathways and the functions of p11 in other cellular processes. Evidence for the role of p11 in the modulation of pain related behaviours in rats particularly in NGF-induced inflammatory conditions is also presented.

At present, there are limited methods available to study the function of p11 *in vivo*. There are no known pharmacological blockers of p11 and no transgenic knock-out animals (conditional or otherwise) have been engineered. The available HSV-derived vectors investigated in chapter two did not achieve a high enough level of transduction; precluding their use to reduce the level of p11 (which is widely and abundantly expressed in nervous tissue). We therefore chose to utilise an antisense approach to try and reduce the level of p11 in sensory cells of the peripheral system.

The use of antisense technology has been used previously in a number of studies which demonstrated appreciable changes in nociceptive-related behaviours after application. The ease of uptake of these molecules remains a key limitation to their wider use both experimentally and therapeutically. Data presented within demonstrates that the application of short (non-thiolated) oligodeoxynucleotides directly into the cerebrospinal fluid allows the efficient transduction (>80%) of DRG neurones.

3.2 Introduction

The 11kDa protein p11 also known as S100A10; annexin II light chain; calpactin I light chain; CAL12; CLP11; p10; CA[1]; or 42C belongs to the S100 family of calcium-binding proteins. So far 20 members of this family have been identified. They have been shown to be involved in a diverse range of intracellular and extracellular activities. These include cell differentiation, signal transduction, cytoskeletal assembly, regulation of protein phosphorylation and transcription (Donato, 2003).

The S100 protein family is characterised by the small size of its members (M_r between 9 - 14kDa) and the presence of two consecutive EF hands (calcium-binding motifs) (Figure 3.1). These are connected by a linker region and flanked by hydrophobic NH_2^+ and $COOH^-$ terminal extensions. These terminals are responsible for the target protein interactions. Generally, S100 proteins can form homo or heterodimers and bind 4 Ca^{2+} ions per dimer. However, p11 (S100A10) has lost the ability to bind Calcium due to mutations in its two EF hands, rendering it unable to bind to Ca^{2+} ions (Gerke and Moss, 2002).

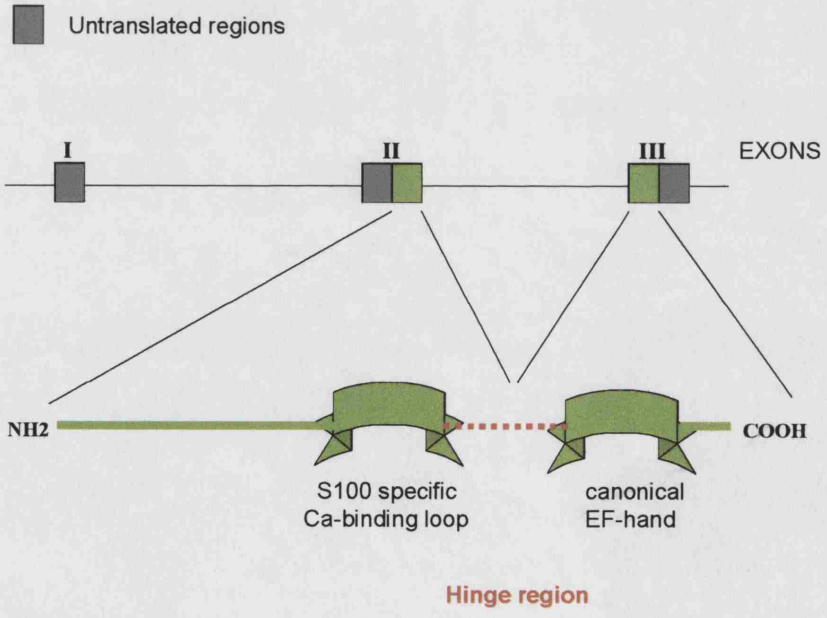


Figure 3.1 Generalized S100 gene and protein structure

A member of the annexin family of proteins, Annexin II heavy chain (also known as calpactin I, p36 or lipocortin II) has also been shown to bind to (Kube et al., 1991; Gerke and Weber, 1984) and regulate expression of p11 (Puisieux et al., 1996). This family of proteins is widely distributed being found in the majority of mammalian cells as well as in some lower organisms, moulds and plants. The annexin family of proteins, which bind to negatively charged phospholipids in a Ca^{2+}-dependent manner, is characterised by many repeats of a highly conserved 70-amino acid domain within its sequence. These proteins are also highly conserved structurally. There are important differences in the N-terminal region of the annexin proteins however, which may contribute to their varied intracellular and extracellular functions (Gerke and Moss, 2002; Moss and Crumpton, 1990; Smith and Moss, 1994).

The interaction between Annexin II heavy chain and p11 occurs due to the presence of certain domains on the N-terminal region of Annexin II heavy chain polypeptide. Annexin II heavy chain exists as a 36kDa cytosolic or nuclear localised monomeric form, in addition to the cytoskeletally-associated heterotetrameric form with p11. This heterotetramer consists of two molecules of annexin II heavy chain bound to two molecules of the p11 protein (Bianchi et al., 1992). The existence of annexin II in various splice variants within the cell may help to explain its diverse range of actions (Upton and Moss, 1994; Smith and Moss, 1994). These include v-Src mediated cell transformation (Osborn et al., 1988), tenascin-C cell to cell adhesion (Harder and Gerke, 1994), interactions with both endocytotic and exocytotic vesicles(Bitto and Cho, 1999) and as a receptor for cytomegalovirus and tissue plasminogen activator (Moss, 1997). The annexin II/p11 complex is present in many tissues and cell lines derived from higher vertebrates (Gerke and Moss, 2002) and seems to be concentrated at the cytoplasmic face of the plasma membrane (Gerke and Weber, 1984; Greenberg and Edelman, 1983).

The formation of the heterotetrameric complex between annexin II heavy chain and p11 seems to be a prerequisite for the efficient association of the complex with the submembraneous cytoskeleton (Thiel et al., 1992). Disruption of this process, either with carboxymethylated p11 which is no longer able to bind to annexin II heavy chain proteins (Osborn et al., 1988) or mutant Annexin II which is unable to bind to p11 (Thiel et al.,

1992) leads to a build up of the complex in the cytoplasm and a decrease in the submembraneous component.

3.2.1 Annexin II and p11

P11 has also been shown to modulate the biochemical activity of annexin II. The complex, but not the monomeric form of annexin II, has the ability to aggregate chromaffin granules at micromolar Ca^{2+} concentrations (Drust and Creutz, 1988) and also to restore Ca^{2+}-evoked exocytosis in permeabilized chromaffin cells (Sarafian et al., 1991).

Although p11 contains mutated EF hands (Figure 3.1) and can no longer bind to Ca^{2+}, its ability to bind to annexin II heavy chain is unaffected (Drust and Creutz, 1988). Indeed, p11 appears to have become a highly active molecule due to its unusual open conformation, allowing its binding sites to be permanently accessible. The conformational change required by most of the S100 family of proteins to expose the active sites on the molecule is usually modulated by Ca^{2+} ions, p11 however does not require Calcium binding to activate it and exists in a permanently active state (Rety et al., 1999).

P11 binds readily to a variety of intracellular proteins and modulates their activities (e.g. cytosolic phospholipase A2 [cPLA2] (Wu et al., 1997); Cyclin-dependant kinase (Cdk)-like protein (PCTAIRE-1) found mainly in the mammalian brain (Sladeczek et al., 1997); Bcl-xL/Bcl-2-associated death promoter (BAD) (Hsu et al., 1997); TASK-1 potassium channel (Girard et al., 2002); epithelial Ca^{2+} channels TRPV5/TRPV6 (Van den Graaf et al., 2003). P11 has also further been shown to bind to and regulate extracellular processes involving plasminogen or plasminogen/annexin II heavy chain complex (Donato, 2001).

The annexin II heavy chain binding sites on p11 have been determined by studies which have progressively truncated the protein to determine the relevant residues involved. The binding site has been located to a highly hydrophobic region located within the C-terminal extension (residues 85-91) (Kube et al., 1992). The formation of the complex is regulated by protein kinase C phosphorylation in the N-terminal domain of the annexin II heavy chain (Gerke and Moss, 2002) and is calcium independent (Rety et al., 1999).

The co-expression of annexin II heavy chain and p11 occurs in a wide range of tissues but the proportion of the monomeric to the heterotetrameric form may vary according to the cell type. In the intestinal epithelium approximately 100% of the annexin II protein occurs as the heterotetramer whereas in cultured fibroblasts up to 50 % exists as free monomers (Zokas and Glenney, Jr., 1997). This observation may be important in determining the function of the p11/ annexin II complex in the cell or tissue under investigation.

Annexin II heavy chain is expressed in most dorsal root ganglia (DRG) neurones and their axons and is present in substantial quantities in mature small-diameter neurones (Naciff et al., 1996). Annexin II expression is also widespread in the developing mouse nervous system with annexin II expression persisting in neurones well into adulthood, lending credence to the possibility that it plays a functional role in mature neurones, as well as during their development (Hamre et al., 2003).

The annexin II/p11 complex appears to influence the formation of cholesterol-rich plasma membrane rafts that are a prominent component of these areas (Harder and Gerke, 1994; Sagot et al., 1997). It may also help to organize actin within these areas leading to a greater stability of the lipid rafts through contacts formed with the cytoskeleton, reviewed in (Gerke and Moss, 2002). The binding of p11 to annexin II heavy chain leads to an enhanced ability of annexin II to bundle f-actin and serves as the docking ligand in the complex, allowing the complex to interact with the membrane (Sagot et al., 1997). The enhanced f-actin bundling activity of the complex in the membrane is due to the inhibition of phosphorylation of the annexin II heavy chain by p11, a steric effect, and an increased affinity for lipid and calcium by the complex compared to the monomeric annexin II form (Ayala-Sanmartin et al., 2000). The increased affinity for lipids is probably due to the bound p11 masking certain negative regulatory elements present on the annexin II heavy chain N-terminal tail. The existence of regions with antagonistic effects with regard to membrane binding and aggregation ability on the N-terminal region of Annexin I have also been observed (Wang and Creutz, 1994; Bitto and Cho, 1999).

Many annexins have also been shown to self associate on membrane surfaces (Lambert et al., 2000), this may be an important mechanism in mediating local concentrations of cell-surface receptors and other membrane proteins and general raft recruitment (Babiychuk and Draeger, 2000).

3.2.2 Interactions of p11 with other proteins

P11 also interacts with other intra and extracellular proteins. Using the C-terminal region of $cPLA_2$ (cytosolic phospholipase A_2) in a yeast-two hybrid screen, (Wu et al., 1997) p11 was identified as an interacting protein. $cPLA_2$ is an enzyme that degrades phospholipids leading to the production of free fatty acids and lysophospholipids. These serve as intracellular messengers or may be metabolised to potent inflammatory mediators such as leukotrienes, prostaglandins, eicosanoids and other metabolites of arachidonic acid. Arachidonic acid (AA) is released from membranes by the action of phospholipase A_2 (PLA_2) and it has been shown that an association between p11 and $cPLA_2$ leads to an inhibition of the PLA_2 *in vitro.* By introducing an antisense oligonucleotides to p11 (in a plasmid) Wu et al (1997) were able to reduce the level of p11 expression on a stably p11 transfected cell line. The reduction in p11-protein resulted in an increase in the activity of PLA2 and a consequent increase in AA release. P11 alone was necessary for this process, as annexin II heavy chain was unable to cause a similar effect on its own (Wu et al., 1997).

P11 has been shown to affect the translocation of the background K^+ channel TASK-1 (Girard et al., 2002). The channel TASK-1 is widely distributed. It is found in the heart, brain, placenta, pancreas, lung, kidney, ovary, prostate and small intestine. In its native form it possesses a sequence of three amino acid residues; ser-ser-val (SSV) as its last three components of the C-terminal region. This SSV motif has been determined as the binding site for p11. When p11 binds to this sequence it masks an area of TASK-1 which contains an endoplasmic reticulum (ER) retention signal. This allows the protein to leave the ER and migrate to the plasma membrane allowing the channel to mediate potassium currents. Disruptions in this process, either though mutations in the SSV p11 binding motif or total cleavage of the signal, disrupts the p11 interaction thus continuing the TASK-1 protein retention in the ER. As described above the p11/annexin II complex has the ability to bind F-actin (Filipenko and Waisman, 2001), however Girard et al (2002) showed that this is not the mechanism of action of channel shuttling to the membrane as the TASK-1 current densities in TASK-1 transfected COS cells was unaffected by cytochalasin-D, which disrupts actin filaments.

P11 has also been shown to interact with the tetrodotoxin-resistant sodium channel $Na_v1.8$/SNS (Okuse et al., 2002). Use of the N-terminal intracellular domain of $Na_v1.8$, amino acids 1-127, in a yeast-two hybrid system identified p11 from a rat P1 DRG cDNA library. A functional role for p11 in the regulation of Nav1.8 channels was then shown using an antisense construct to p11 introduced into sensory neurones in culture. The efficient knock-down of p11 protein (shown by immunohistochemical means) was mirrored by a great loss in $Na_v1.8$ current. Conversely, introduction of p11 into a cell line which expresses $Na_v1.8$ protein but not its associated current showed an increase in the $Na_v1.8$ current. It is postulated that p11 mediates the channel insertion into the plasma membrane. It is also possible that the channels associated with p11 may become locally abundant in lipid rafts through interactions of p11 with annexin II (Okuse et al., 2002; Malik-Hall et al., 2003; Poon et al., 2004).

3.2.3 Nerve growth factor and p11

Nerve growth factor (NGF) is one of the best characterised polypeptide growth factors. NGF is responsible for the selective survival of populations of sympathetic and some sensory neurones during development (Aloe et al., 1992). It is also important in maintenance of the differentiated state of these neurones thereafter. NGF has an interesting effect on rat adrenal pheochromocytoma-derived cells (PC12 cell line). These cells normally show the characteristics of adrenal chromaffin cells, but upon exposure to NGF, they acquire the properties of sympathetic neurones (Garber et al., 1989). These changes include the development of neurites up to 1mm long in a process which is thought to mimic the developmental pathway of embryonic neuroblasts responsive to NGF.

In 1988, Masiakowski *et. al.* also showed that the mRNA for p11 in PC12 cells is upregulated upon long exposure to NGF (Masiakowski and Shooter, 1988). Treatment with NGF was also demonstrated to lead to an increase in the levels of p11 mRNA in cultured DRG neurones (Okuse et al., 2002). The application of NGF to PC12 cells (Garber et al., 1989) as well as cultured DRG neurones (Fjell et al., 1999) has also been shown to increase the sodium current density. The activity of p11 with regard to $Na_V1.8$ may therefore be to increase the trafficking of $Na_V1.8$ protein to the membrane, under conditions where there is an elevated concentration of NGF (such as inflammation). The

disruption of this link may provide a means of addressing NGF-related pain a component of the inflammatory response.

3.3 Objectives

This study undertook to determine whether an antisense approach to the removal of p11 could be achieved effectively with non-thiolated short oligonucleotide sequences directed against the p11 sequence and whether modifications in pain-related behaviour could be elicited. In particular, given the importance of p11 in regulating the $Na_V1.8$ system, a model of pain which is dependant on $Na_V1.8$ (NGF-induced inflammation) was tested to determine the possible modulation in pain-behaviours associated with this condition (Kerr et al., 2001). NGF has already been shown to modulate $Na_V1.8$ mRNA levels in cultured cells (CHO-SNS) (Okuse et al., 1997). The upregulation of p11 by NGF (Masiakowski and Shooter, 1988) has also been shown. Therefore, further evidence of the regulation of $Na_V1.8$ levels in various cellular regions such as the membrane and cytosol by p11 was sought by determining the levels of $Na_V1.8$ protein present in various subcellular fractions *in vivo* before and after treatment.

NGF has also been shown to modulate pain in both the gut and bladder (Yoshimura et al., 2001b) in a similar manner to peripherally mediated pain states. This process also involves the regulation of $Na_V1.8$ channels. The consequence of p11 ablation in the DRG on bladder function was therefore also investigated using cystometric measures. TTX-resistant currents have also been recorded in enteric ganglia (Moore et al., 2002; Rugiero et al., 2003) and some DRG neurones also send projections into the gut (Laird et al., 2002). The modulation of p11 may therefore also participate in the modulation of these visceral pain pathways in a similar manner to that proposed for NGF induced inflammatory pain states.

3.4 Methods and materials

3.4.1 P11 antisense studies *in vivo*

Regions of the murine p11 mRNA sequence (accession number NM009112) were chosen to create short antisense oligonucleotides by alignment of the S100 family of proteins and selecting stretches of sequence which were unique to p11 (S100A10). These sequences were not modified with in any manner, as the common modifications such as thiolating various residues may aid the uptake of the oligo and/or stabilse the sequence against endonucleases, but may affect binding to the target sequence. The AS-1 sequence was designed to be complementary to the sequence from base 100 to 80 and AS-2 from base 126 to 104 (5'-3'). Corresponding mismatch sequences (MM-1 & MM-2) were made for each of the two antisense sequences (AS-1 & AS-2) by switching pairs of adjacent residues (underlined in sequence below). These sequences were then BLAST searched to check for homology with other rat transcripts. No homolgous domains were identified in sequences other than p11. AS-1 and AS-2 binding sequences were found in p11 sequences from a wide range of species including p11 sequences for rat, mouse and human, (all 100 %); and Cow (95 % homology).

p11 AS-1	5'–GTT TCC ATG GCA TGC TCC ATT-3'
p11 MM-1	5'-GTT CTC AGT GCA GTC TCA CTT-3'
p11 AS-2	5'-CAA ACC TGT GAA ATG TAA GCA TC-3'
p11 MM-2	5'-CAA CAC TGT AGA ATT GAA GAC TC-3'

These non–phosphate modified oligonucleotides were obtained from Sigma-Gynosys.eu.com HPLC grade, lyophilized and resuspended in sterile PBS at a concentration of 1μg/μl. They were delivered intrathecally to the animals at a concentration of 10μg/day using a plastic catheter placed sub-durally as outlined below.

3.4.2 Fluorescence analysis

To determine the efficiency of uptake of the delivered oligonucleotides the above oligos were also made with Texas red residues attached to the 3' end. The course of uptake was visualized using a fluorescent microscope. The rat DRG were dissected after 5 days of treatment with oligos delivery intrathecally and fixed in 4% ice-cold fresh paraformaldehyde (PFA) (4% PFA in PBS, pH7.4) for 1 hour on ice. After cryoprotection in 20 % sucrose 4°C o/n, 20μm sections were cut on a cryostat and mounted on slides with citifluor (PBS-Glycerol mixture, Gibco BRL). The degree of uptake was visualized using a fluorescent microscope.

3.4.3 Laminectomy and canulation of animals

Experiments were performed on adult Wistar rats (220-260g). Each animal was fitted with a cannula of polyethylene tubing (PE-10) implanted after a laminectomy at the Th11 vertebra under sterile anaesthetic conditions (4% Halothane/ oxygen mixture). The terminal of the cannula was placed under the dura, directly over the lumbar enlargement area (directly over L3-L4). The distal end of the cannula was passed under the skin of the back and brought out through a hole in the forehead. The cannula was secured by means of glue to the spine and to the parietal region of the skull. The free end of the cannula was sealed with glue to prevent infections. Animals were housed singly post-operatively to prevent cannula displacement and were kept under a 12-hr light/dark cycle (lights on 7:00 am behavioural testing after 8:00 am) with food and water *ad libitum*.

3.4.4 Delivery protocol for antisense molecules

One week post canulation the oligonucleotides were injected. Each day the animals were anaesthetized briefly with a mixture of 4% Halothane/oxygen, the glue removed and 15μl sterile saline delivered first to flush out the cannula followed by the test oligonucleotide (ODN) (10μg in 10μl PBS) followed by 10μl saline flush. The delivery was done once daily for six days. The animals were grouped as follows, AS-1red (n = 5); MM-1red (n = 5); AS-1 (n = 12) MM-1 (n = 12); AS-2 (n = 6); MM-2 (n = 6). An

additional control group receiving saline was also tested (n = 3). Animals were left with indwelling catheters for a total of fourteen days, after which dissections were carried out to determine whether the cannula was positioned at the level of L4-L5 of the spinal cord.

3.4.5 Behavioural testing

After a period of recovery of seven days, the rats were subjected to a daily testing scheme to determine the thermal sensitivity in both rear paws following a double blind design. The Hargreaves method (Hargreaves et al., 1998) was used to measure thermal hyperalgesia using the rat plantar test (Ugo Basile, Comerio, Italy). Rats were habituated for 10-15 minutes to the apparatus which consists of Perspex observation chambers into which the rats are placed upon a glass table. A mobile radiant heat source was located under the table and focused onto the rear paw midway between the paw edges level with the base of the thumb joint. Paw withdrawal latencies (PWL) were taken three times for both hind paws with at least 2-minute intervals between each subsequent test. The mean of the three measures represented the baseline for the animals before the start of treatment. The equipment was calibrated to give a mean PWL of approximately 8s.

3.4.5 Nerve Growth Factor (NGF) induced thermal hyperalgesia paradigm

In order to test the effect of the administration on the development of NGF induced thermal hyperalgesia, animals were subjected to five days exposure to the oligos, followed by an injection of 500ng HrNGF (50µl carrier volume) (Human recombinant Nerve Growth Factor 4.2mg/ml in 20mM succinate buffer (pH 7.0) diluted to 10µg/ml with saline) into the subcutaneous plantar surface of the left hind paw. The injection was done under anaesthesia induced by 4% halothane/oxygen to ensure reproducible injections of a consistent position and depth. The animals recovered within 5 minutes. The development of the thermal hyperalgesia was tracked for various time points after the injection, up to a maximum of 24 hours.

3.4.6 Cystometric assessment (bladder function)

Thus study was carried out on female adult Wistar rats (220-260g) because of the easier insertion of the canula into females compared with males. The animals were anaesthetised with urethane (1.25g/kg, *i.p.),* which produced a stable level of anaesthesia during the course of the experiment. This was done 12 hrs after the last ODN injection. The body temperature was maintained at 37°C by means of a heated blanket under the animal. The bladder was catheterized transurethrally with a 1.1mm polythene catheter (PE-50). The correct positioning was assessed by squeezing the bladder and emptying it of urine. The primary measure of bladder motility was assessed by cystometrograms. In this test the bladder is slowly filled with sterile saline solution though an infusion pump (0.05 ml/min for 30 min), the intravesicle pressure slowly increasing (measured by a pressure transducer connected to a side arm of the filling catheter). The rate of filling used is within the physiological range. At some point the micturition threshold (M_T) is reached and a large number of active contractions (micturition contractions) are elicited. The M_T is taken as a measure of bladder motility. The number of contractions within a thirty minute test period (N_C) and the total time of contractions within the test period (T_C) were also recorded. Each animal was tested twice.

3.4.7 Data treatment

Data points were collected for various times daily for four days before the start of the experiment. After these baseline values reachesd a steady, consistent reproducible level the experiment course was started. The behavioural testing was done at 10am followed by the oligonucleotide infusion at 6pm. This was done for four days. On the fifth day 500ng NGF was injected subcutaneously to the left rear paw and data points at 0.5, 1, 2, 4, 7 and 24 hours *p.i.* were taken. Each reading was taken three times and the mean of these values calculated. All animals in a particular treatment group were collected together and the mean plotted. Only animals with properly indwelling catheters for the entire period were used. Paired two-tailed tests of significance were done between control and test groups with a P value > 0.05 taken as significant in all cases.

3.4.8 Culture of CHO-SNS22 cell line

The expression of $Na_V1.8$ protein in a Chinese Hamster Ovary cell line was accomplished by stably transfecting a plasmid containing the full length $Na_V1.8$cDNA under the control of a CMV promoter into the CHO cells (Okuse et al., 2002; Okuse et al., 1997). This cell line termed CHO-SNS22 expresses abundantly the $Na_V1.8$ protein but it is located almost exclusively in the cytoplasm. The total cell proteins extracted from two 6cm dishes were used to prepare $Na_V1.8$-positive control protein samples. The culture conditions were as follows; The Cells were grown in Nutrient Mixture F-12 (Ham) media (Gibco BRL) supplemented with 2.5% final concentration (v/v) foetal bovine serum + 1% final concentration (v/v) Penstrep (penicillin/streptomycin from Sigma) + 1mg/ml G418 (selection agent to prevent loss of the plasmid) until 80% confluent (media changed daily, 5% CO_2, humidified incubator 37°C). The cell media conditions were then changed to reduce the foetal bovine serum component to 0.5% and the cells cultured for an additional two days. This step was necessary to stress the cells further (in addition to the confluence-induced strain) to induce the large-scale production of $Na_V1.8$ protein. The cells were harvested with 3ml trypsin/plate (Sigma) and recovered for protein extraction by centrifugation at 1500 rpm, 10 min and at room temperature. The pellet was immediately frozen at -80°C to prevent degradation.

3.4.9 Western blot to determine changes in $Na_V1.8$ protein levels in DRG

In order to determine the effect of p11 antisense oligos on the levels of $Na_v1.8$ protein present in various subcellular fractions, Western blot analysis was carried out on freshly dissected DRG obtained from the treated rats. This was done as follows:

3.4.9.1 Tissue harvest and preparation

Dissections of the rats were done quickly on ice (less that 15 min) and the extracted DRG snap frozen in liquid nitrogen. They were then stored at -80°C until further processing to prevent degradation of the proteins by endogenous proteases. The ganglia

from lumbar region 3-6 (L3-L6) were harvested, both ipsilateral and contralateral to the site of NGF mediated inflammation.

3.4.9.2 Separation of cell fractions

The harvested tissues were homogenised in a 1ml mechanical Dounce glass homogenizer on ice. The DRG (8 per extraction, two animals pooled) were placed in 250µl of ice-cold extraction buffer containing protease inhibitors and detergents. (Extraction buffer; PBS + 1%NP40; 2% Triton-X100; 0.5% Na Deoxycholate; 4% SDS; 5mM PMSF; 2mM Benzamidine; 1mM iodoacetamide; 50mg/ml aprotinin;1µl protease inhibitor cocktail [Sigma p8340]; pH7.4). Using twenty stokes of the homogenizer the tissue was broken up and incubated for 20 minutes on ice. Care was taken to avoid bubbles in the mix. The homogenate was sonicated for 5 X 5 seconds with cooling on ice in-between each treatment to help the $Na_v1.8$ protein to go into solution. The homogenate was centrifuged at 5000 rpm (2000 x g)/10 min/ 4°C to remove the nucleus and cell organelles such as mitochondria. The pellet was discarded. Samples of the supernatant were taken to represent the whole cell contents. The supernatant was then partitioned into two fractions using an ultracentrifuge. The supernatant was spun for 6 hours at 40,000 rpm (200,000 x g)/ 4°C to sediment the membrane fraction. The resultant pellet was resuspended in 200µl extraction buffer and represented the membrane fraction of the cell. The supernatant (approx. 200µl) composed the cytosolic fraction. All samples were stored at -80°C until use.

3.4.9.3 Separation of proteins

The samples were analysed to check the amount of proteins contained in each sample using a UV spectrophotometer using the UV-absorbance (A280) method (www.science.smith.edu/departments/biochem/biochem_353), and equal amounts (5µg per lane) of each sample to be tested were loaded on an 8% SDS-PAGE acrylamide gel. Samples were incubated at 100°C for 10 minutes with SDS-Loading buffer and 10µl Marker (Amersham RPN756 rainbow marker) loaded for comparison. As a positive control, 5µg of Protein extracted from a CHO-SNS expressing cell line. The gel was

electrophoresed at 200A o/n 4°C (running buffer; 1 X Laemmli; 3.03% (w/v) Tris base + 14.42% (w/v) glycine + 1% (w/v) SDS in dH_2O, pH 8.3). The size expected for $Na_V1.8$/SNS was 260kDa. Duplicate lanes were produced on each gel so that the duplicate gels could be checked for protein levels contained in the gel with Coomassie brilliant blue stain (data not shown) (fix solution; 50% Methanol + 10% acetic acid + 40% dH_2O, 15 min, room temperature), (stain solution; 0.04% (w/v) Coomassie Brilliant Blue R-250 + 10% acetic acid + 25% isopropanol in dH_20, o/n, room temperature), (destain solution; 10% methanol + 7.5% acetic acid in dH_2O, 2hrs, room temperature) before transfer to nitrocellulose paper. The duplicate non-stained gel for each preparation was transferred to Hybond N nitrocellulose membrane (transfer buffer; 20mM Tris pH 8.3; 0.01% (w/v) SDS; 240mM glycine; 20% methanol) 48 hours, 4°C, 200A).

3.4.9.3 Detection of $Na_V1.8$ protein

After transfer of the proteins to the nitrocellulose membrane (Hybond Super ECL, Amersham Life Science, Buckinghamshire, UK) a polyclonal antibody was used to detect the presence of $Na_V1.8$ protein (SNS poly 11, raised against the C-terminal region). First the membrane was blocked with 5% non-fat milk proteins in PBT-T (1 X PBS + 0.1%v/v Tween-20, 30 min room temperature, agitation). After three washes in PBS-T, 5 min, room temperature with shaking, the membrane was probed with 1:500 polyclonal anti-SNS antibody in PBS-T followed by a further three washes in PBS-T. The secondary 1:400 horse-radish peroxidase conjugated anti-rabbit antibody was applied for 1 hour, room temperature followed by three washes in PBS-T as before. Detection of the bound secondary was done using ECL high sensitivity reagent as described in the supplied protocol. The Horse-radish peroxidase catalyses the oxidation of luminal in alkaline conditions (in the presence of phenol). With oxidation, the luminal molecule is excited and upon returning to ground state emits light of the 428nm wavelength. This is detected by the radiography film. Exposures were made in the 5-10 s range on KODAK Biomax MS-1 film.

3.4.10 Densitometric analysis of $Na_V1.8$ protein bands

The density of the bands obtained was taken as a measure of the amount of $Na_V1.8$ protein present in the sample. The bands were quantitated using the public domain NIH image program (http://rsb.info.nih.gov/nih-image). In each case the density for the band area was normalized by subtracting an equivalent area of background occurring next to the band. Six pairs of measurements were taken for each band encompassing the entire area. These six values were then averaged to give the final estimate of density of the band ± S.E. The results were plotted on a percentage greyscale to indicate the relative intensities measured. The higher the percentage the more dense the original band appeared on the film (i.e. a darker band indicating more protein).

3.5 Results

3.5.1 Intrathecal delivery of short oligonucleotides to DRG *in vivo*

The delivery of a Texas-red labelled (non-phosphate modified) oligonucleotide was used to determine the efficiency of uptake of the ODNs by the rat DRG cells *in vivo*. The oligonucleotide sequences around 21-24 bases long were synthesized with a Texas-red dye molecule attached to the 3' terminal. An intrathecal cannula was used to infuse the ODNs into the cerebrospinal fluid. 24 hours after a single dose of 10μl of 10μg/μl oligonucleotide efficient uptake of the ODN can be visualised in DRG cells (Figure 4.2). Approximately 80% of cells (small and large diameter) in the DRG ganglia located in the lumbar region absorbed the oligonucleotides (n = 6 DRG from 3 animals counted).

3.5.2 Affect of infused antisense oligonucleotides on baseline levels of thermal sensitivity *in vivo*

The Hargreaves method of assessment of the thermal hypersensitivity was used. All animals were of the same sex (female), age (adult) and weight (~ 250g). The base line values obtained before and after five days infusion were unchanged (naïve, 8.20 ± 0.16 s; saline only, 8.23 ± 0.27 s; MM-1, 8.08 ± 0.26 s; MM-2, 8.05 ± 0.20 s; AS-1, 8.01 ± 0.28 s; AS-2, 8.01 ± 0.33 s; n = 6, $P > 0.1$; Paired two-tailed t-test). There were no other apparent behavioural or physiological changes to the treated animals during the course of the study.

3.5.3 Hyperalgesic effects of subcutaneous NGF in the presence of test oligonucleotides *in vivo*.

The injection of 50μl of carrier solution containing 500ng Hr-NGF created a small pocket under the subcutaneous plantar surface of the left hind paw, which faded after 30 minutes. Testing in the area of the injection was avoided. The dose of 500ng NGF/paw elicited a marked thermal hyperalgesia in the ipsilateral but not contralateral paw, which persisted until the end of the experiment 24 hrs later (Figure 3.3). This is in accordance

with the published literature (Lewin et al., 1994; Andreev et al., 1995) In agreement with this group's findings the animals displayed a biphasic response to the NGF. An acute phase of hyperalgesia beginning 30 minutes into the testing period with some recovery at 2 hours followed by a second period of hyperalgesia which appeared around 3 hours. The hyperalgesia observed persisted until the end of the experiment.

The development of the hyperalgesic response was not impaired in the ipsilateral paw by the presence of the control oligonucleotides, which were shown by blast searches to have no homology with any known rat sequences (no significant difference between MM-1 treated and saline control; $n = 5$, $P > 0.1$; Paired two-tailed t-test), (Figure 3.3).

The contralateral paw (right rear) also showed no significant change to Hargreaves test of thermal hyperalgesia in any of these studies (naïve, 8.2 ± 0.6 s; after NGF 2hr, 8.1 ± 0.6 s; after NGF 4 hr, 8.0 ± 0.5 s; $n = 10$, $P > 0.1$; Paired two-tailed t-test). This demonstrated that the dose of NGF administered was sufficiently small to prevent widespread systemic effects.

3.5.4 Antisense AS-2 vs mismatch control MM-2 effects on NGF-mediated thermal hyperalgesia

Administration of AS-2 or its mismatch pair oligonucleotide MM-2 had no statistical effect on the development of the NGF evoked thermal hyperalgesia in the ipsilateral (left rear) paw (Figure 3.5) when compared with each other. AS-2 was designed to be a 21-mer antisense oligo directed against bases 80-100 of the N-terminal region of the P-11 coding region and contained the start ATG codon. ($P > 0.1$; Paired two-tailed t-test).

3.5.5 Antisense AS-1 vs mismatch control MM-1 effects on NGF-mediated thermal hyperalgesia

Administration of AS-1 had a statistically significant effect on the development of the NGF evoked thermal hyperalgesia in the ipsilateral (left rear) paw compared with its mismatch control MM-1 (Figure 3.4). AS-1 was designed to be a 23-mer antisense oligo directed against bases 104-126 of the N-terminal region of the P-11 coding region. The administration of antisense oligonucleotides directed against this region of the N-terminus

of p11 (AS-1) lengthened the latency time before paw withdrawal for three time points after the administration of NGF compared with the control (MM-1 treated animals). The mean withdrawal time for the AS-1 treated animals for the 0.5 hr, 1hr and 7 hr are as follows; 0.5 hr, 9.9 ± 0.4 s; 1 hr, 10.0 ± 0.4 s; 7 hr, 6.7 ± 0.2 s, compared to the MM-1 treated animals; 0.5 hr, 5.7 ± 0.3 s; 1 hr, 4.4 ± 0.1 s; 7 hr, 4.3 ± 0.2 s, (mean ± s.e.m.; n = 7, $P < 0.05$; Paired two-tailed t-test). This result shows that in the animals treated with the AS-1 antisense oligo to p11, the acute phase of sensitization due to the peripheral application of NGF was nearly abolished only appearing after the two hour time point with a rapid recovery by four hours and the subsequent phase was reduced in intensity (36 ± 5 % reduction) compared to the control response.

3.5.6 Bladder motility

The assessment of bladder motility was done to determine the effect of the oligonucleotide treatment on bladder function as it has been shown that $Na_V1.8$ channels present in the bladder afferents are important in mediating visceral pain (Yoshimura et al., 2001b). This experiment was carried out to establish if the afferent fibres originating in the DRG were responsible for the transmission of visceral pain.

There were no statistically significant changes in micturition threshold (M_T), number of super-threshold contractions (N_C) or total time of contraction within a 30 minute run (T_C), upon application of any of the test nucleotides. AS-1 (n = 7) (M_T, 13.1 ± 3.4 min; N_C, 12.5 ± 3.7; T_C, 284.8 ± 68.2 s) compared with MM-1 control (n = 5) (M_T, 12.6 ± 4.1 min; N_C, 13.6 ± 4.6; T_C, 308.2 ± 82.7 s) (Figure 3.6) or AS-2 (n = 5) (M_T, 13.7 ± 1.0 min; N_C, 12.8 ± 2.4; T_C, 521.3 ± 73.4 s) compared with MM-2 control (n = 5) (M_T, 15.9 ± 5.0 min; N_C, 19.3 ± 9.3; T_C, 524.5 ± 175.7 s) Student's two-tailed heteroscedastic t-test $P >$ 0.1 (Figure 3.7).

3.5.7 Western blot analysis of $Na_V1.8$ protein levels

The effect of administration of 5-days of either AS-1 or MM-1 ODN's on the $Na_V1.8$ protein levels and subcellular distribution were compared to control untreated levels by means of Western blots.

The level of protein expressed in the untreated controls was quite low. This reflected the difficult in solubilizing $Na_V1.8$ protein and the limited amount of starting tissue (8 DRG per prep). In the normal situation (Figure 3.8 control untreated), $Na_V1.8$ protein, as shown by a band of > 250kDa (approximately 260 kDa) can be detected in the whole cell extract (minus nucleus, Golgi body and other large cell organelles), it was also readily apparent in the cytosolic fraction but much reduced in the membrane fraction.

After treatment with NGF the densitometrically determined level of $Na_V1.8$ protein expressed in the ipsilateral DRG went up by approximately 53.2 ± 4.7 % in the whole cell extract, 42.8 ± 6.8% in the cytosolic fraction, 78.8 ± 6.3% in the membrane fraction compared to control levels (n=6) after normalization for variations in background.

When the animals were treated with NGF following MM-1 treatment, similar increases on the ipsilateral side were seen compared with untreated control animals; 53.6 ± 7.2% in the total cell extract, 35.4 ± 7.7% in the cytosolic fraction, 76.4 ± 6.9% in the membrane fraction (n=6). These results support the finding that the delivery of MM-1 oligonucleotides had no effect on $Na_V1.8$ protein expression.

On delivery of the AS-1 ODN however, a clear difference could be observe in the localization of the $Na_V1.8$ protein in the various subcellular fractions (Figure 3.9). After NGF treatment the increase in the level of protein in the ipsilateral DRG compared to the untreated control was as follows; whole cell fraction, 41.0 ± 7.8%; cytosolic fraction, 31.9 ± 9.1%; membrane fraction, 4.3 ± 1.7% (n=6). The increases in $Na_V1.8$ protein found in the total cell and the cytosolic component were comparable to those observed in the NGF control and the MM-1 / NGF treated group, however, the drop in $Na_V1.8$ protein located in the membrane fraction in the ipsilateral DRG harvested from the AS-1 treated group after NGF treatment represented a 94.4 ± 5.3% reduction in the levels seen in the NGF control group. These data show that the suppression of p11 expression though the use of directed oligos can affect the sub-cellular localization of mature $Na_v1.8$ protein in DRG cells (Figure 3.10).

Figure 3.2 Efficiency of oligonucleotide uptake in rat DRG after a single direct injection of fluorescent oligonucleotides into the cerebrospinal fluid.

12µm thick section of L5 rat DRG

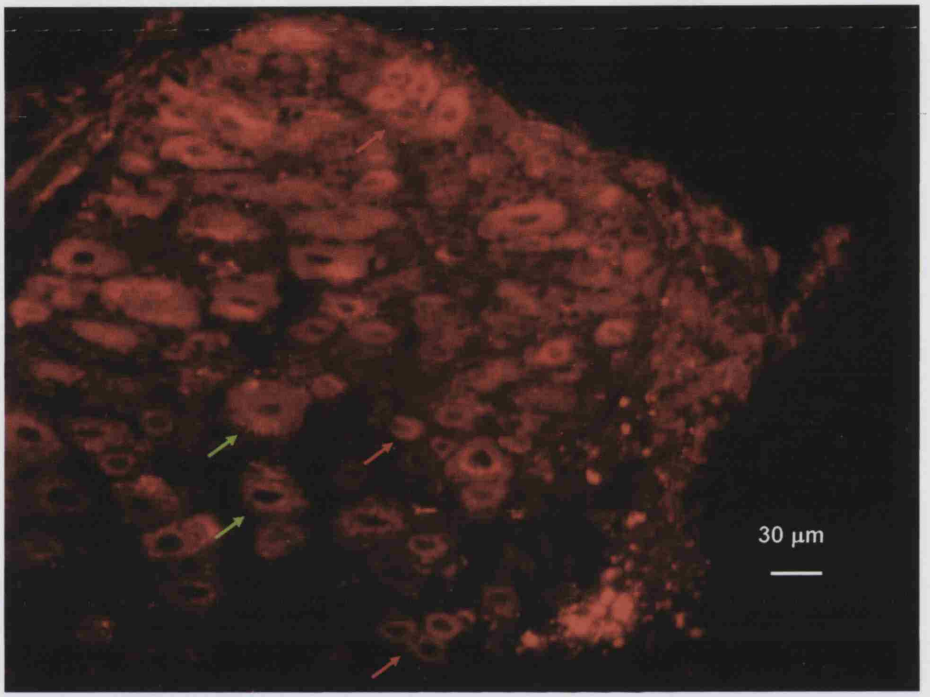


Figure 3.2. Fluorescent microphotograph illustrating the efficient uptake of short oligonucleotides delivered by means of an indwelling cannula; one injection of Texas red-labelled oligos into the cerebrospinal fluid over the lumbar enlargement followed by dissection at 24 hours *p.i.* The high level of fluorescence exhibited above indicates that both large-diameter (green arrow) and small-diameter (red arrow) neurones take-up the antisense oligonucleotides effectively. Non-treated DRG extracted from sham operated controls did not exhibit fluorescence therefore auto fluorescence was minimal (data not shown).

Figure 3.3 Effect of control oligos vs saline on NGF-mediated thermal hyperalgesia (n=5)

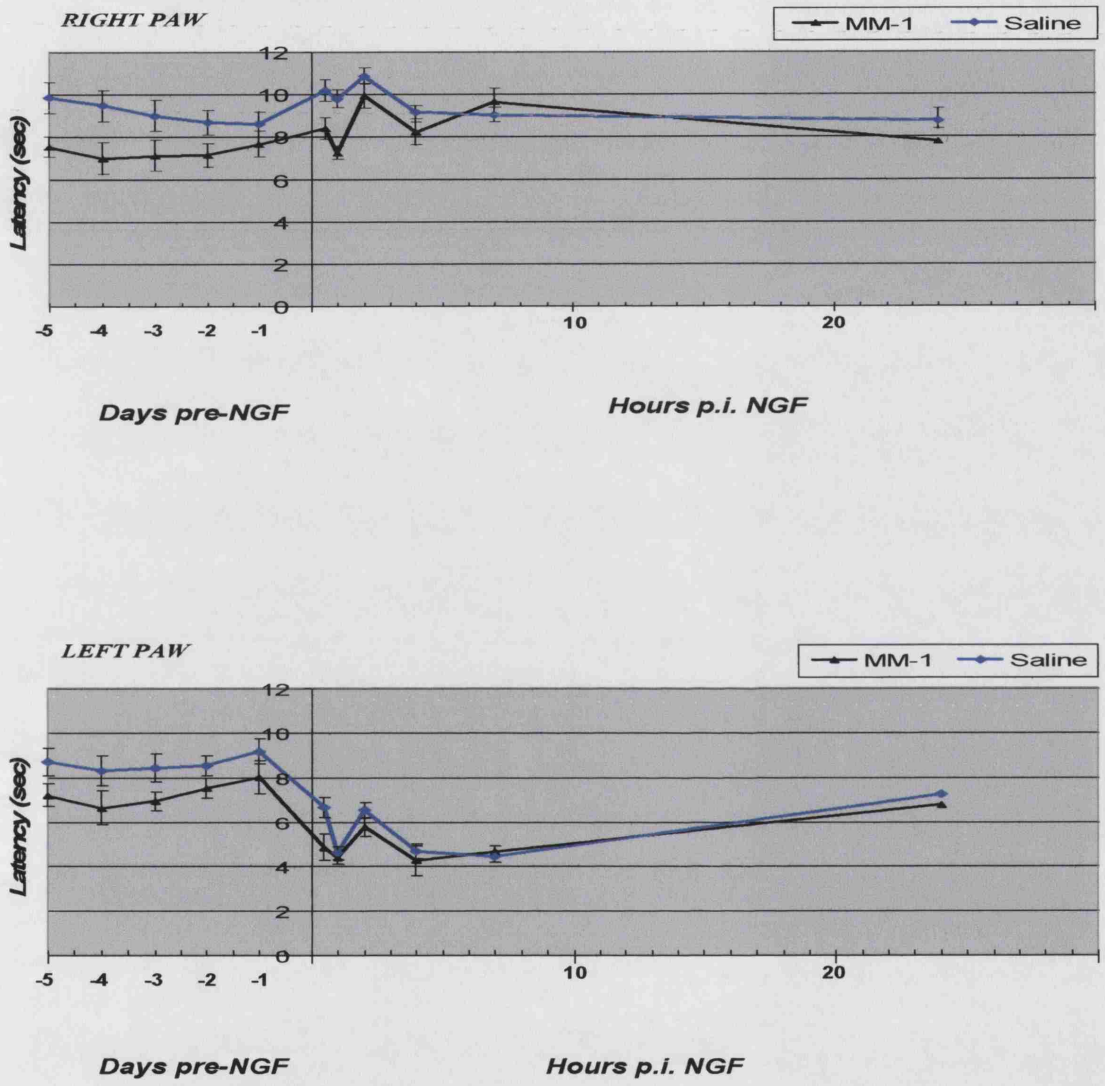


Figure 3.3; No significant effect on paw withdrawal times between animals treated with a non-specific oligonucleotide (MM-1) compared with saline carrier only, both before and after NGF-induced inflammation (added at t=0). Top) contralateral (right paw) response, bottom) ipsilateral (left paw) response. Data presented as means ± SE (n=5)

Figure 3.4 effect of AS-1 vs MM-1 control on NGF-mediated thermal hyperalgesia (n=12)

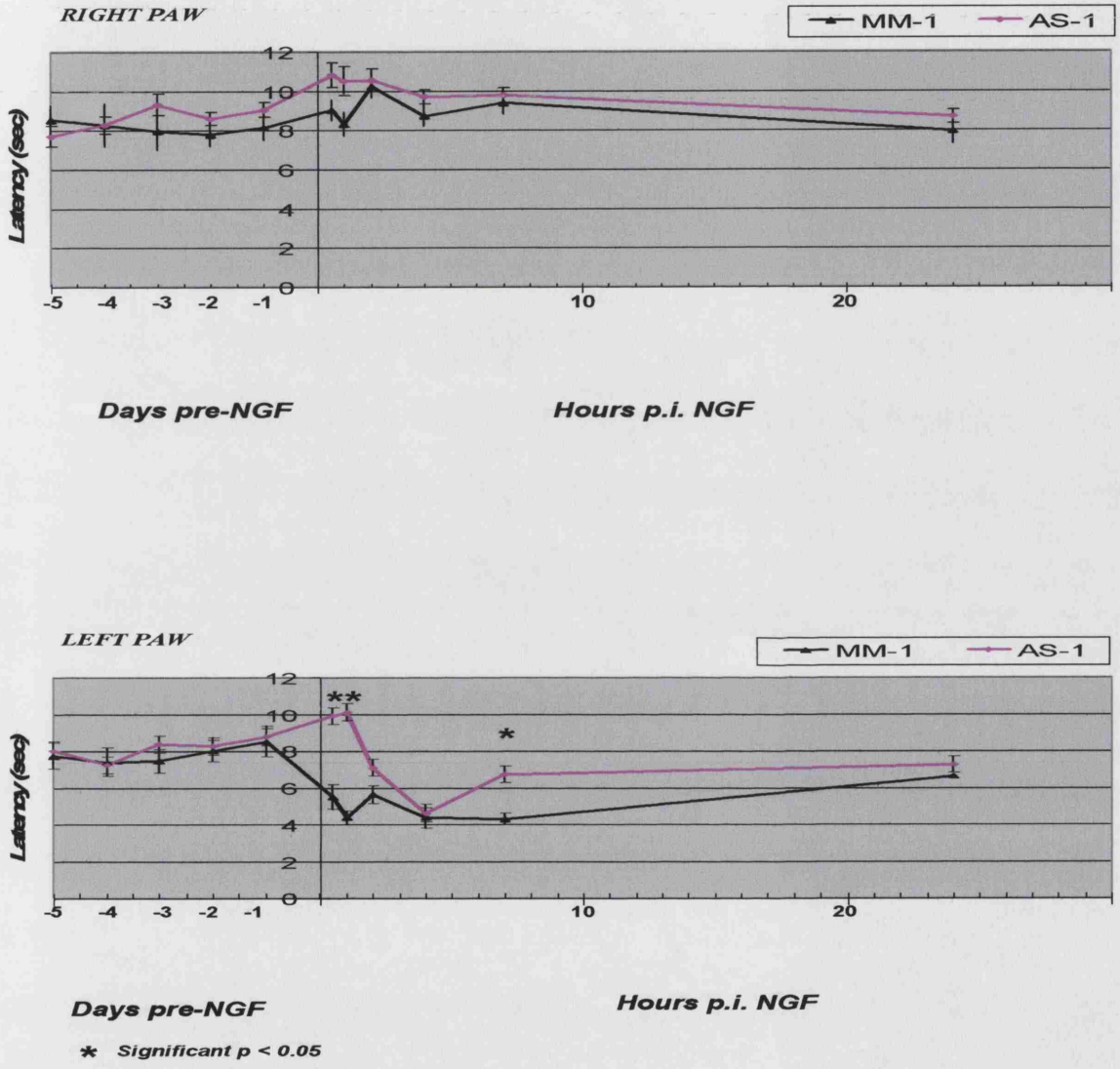


Figure 3.4; the effect of 5 days pre-treatment of AS-1 or MM-1 on paw withdrawal latency times after intra-plantar application of NGF. Top) contralateral response times (right paw), bottom) ipsilateral response times (left paw). The application of AS-1 results in an amelioration of the thermal hyperalgesia which accompanies NGF application (at t = 0) at the 0.5, 1 and 7 hour time points. Data presented as means ± SE (n=12).

Figure 3. 5 Effect of AS-2 vs MM-2 control on NGF-mediated thermal hyperalgesia (n=6)

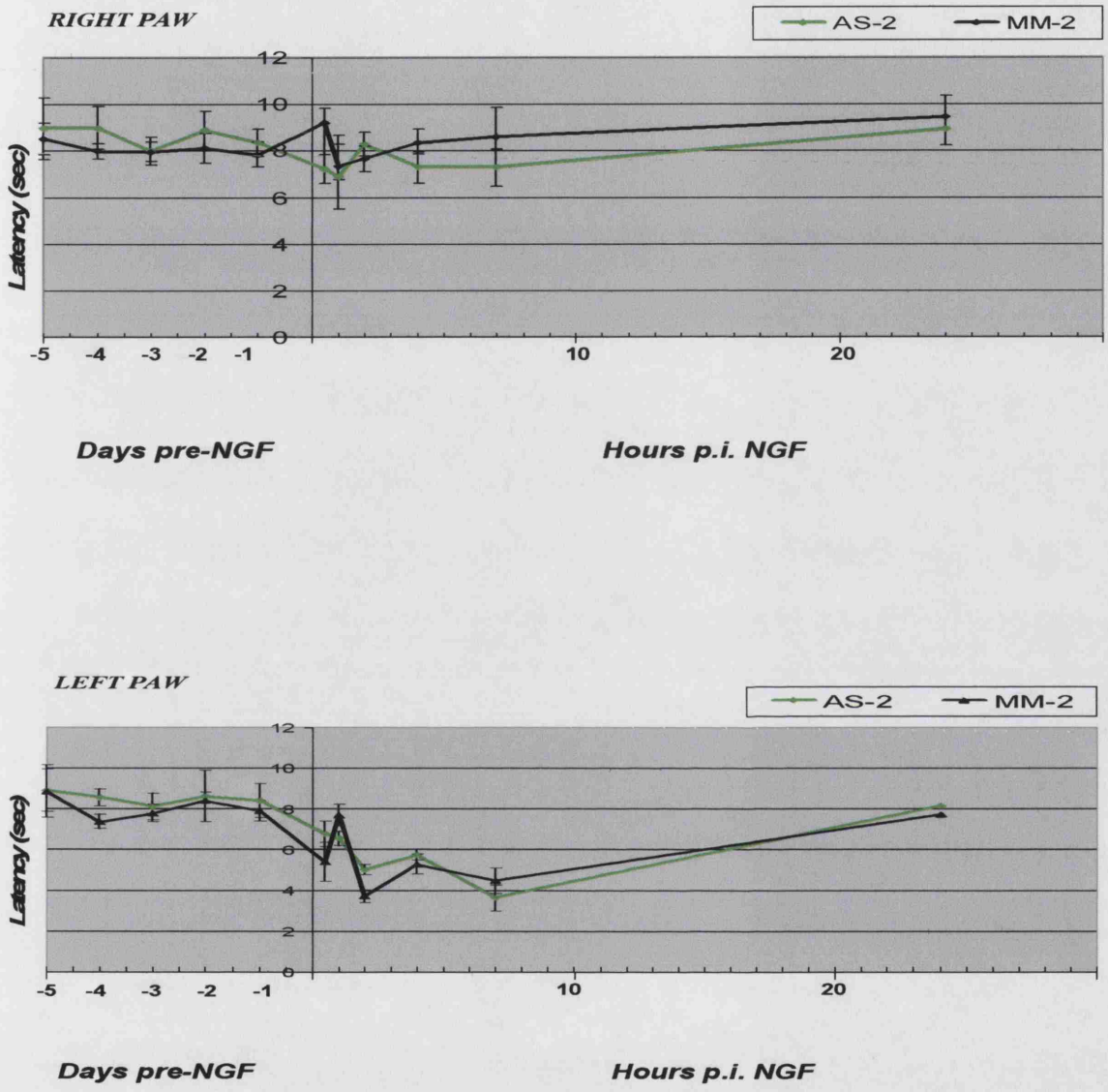


Figure 3.5; The effect of 5 days pre-treatment of AS-2 or MM-2 on paw withdrawal times after intra-plantar application of NGF (at t = 0) in the left paw. Top) contralateral (right paw) response, bottom) ipsilateral (left paw) response. There is no significant difference with the application of AS-2 compared to the MM-2 control Data presented as means ± SE (n=6).

Figure 3.6 Bladder dynamics of rats treated with MM-1 or AS-1 (n=12)

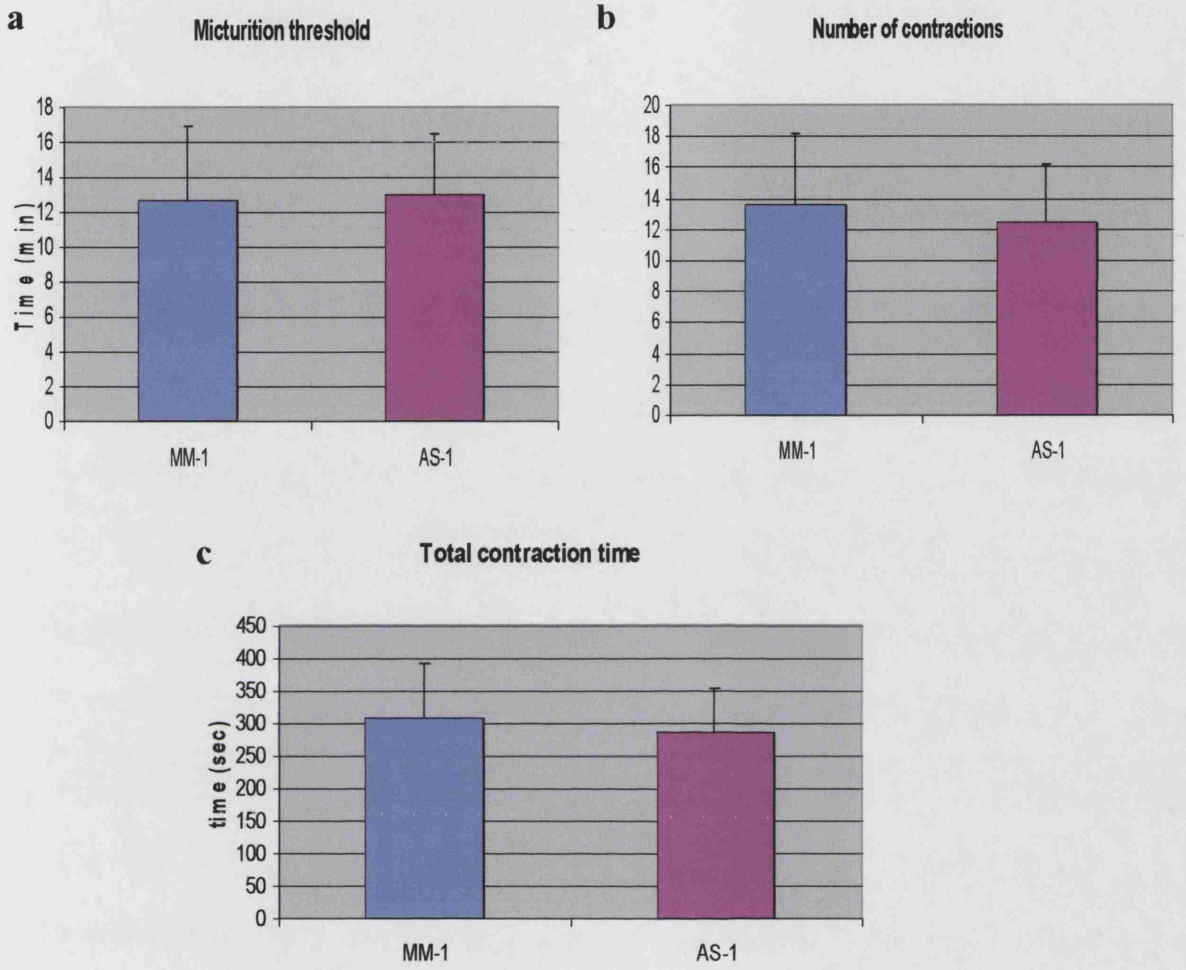


Figure 3.6: No significant difference is observed in the bladder dynamics of the two groups treated with either antisense oligo AS-1 compared to the control mismatch MM-1 oligo.a) the time taken to begin contractions (micturition measure) is the same for both groups; b) the number of contractions exhibited is not significantly different; c) The total time during which contractions are exhibited is also unchanged. Data presented as means ±SE (n = 12).

Figure 3.7 Bladder dynamics of rats treated with AS-2 vs MM-2 (n=12)

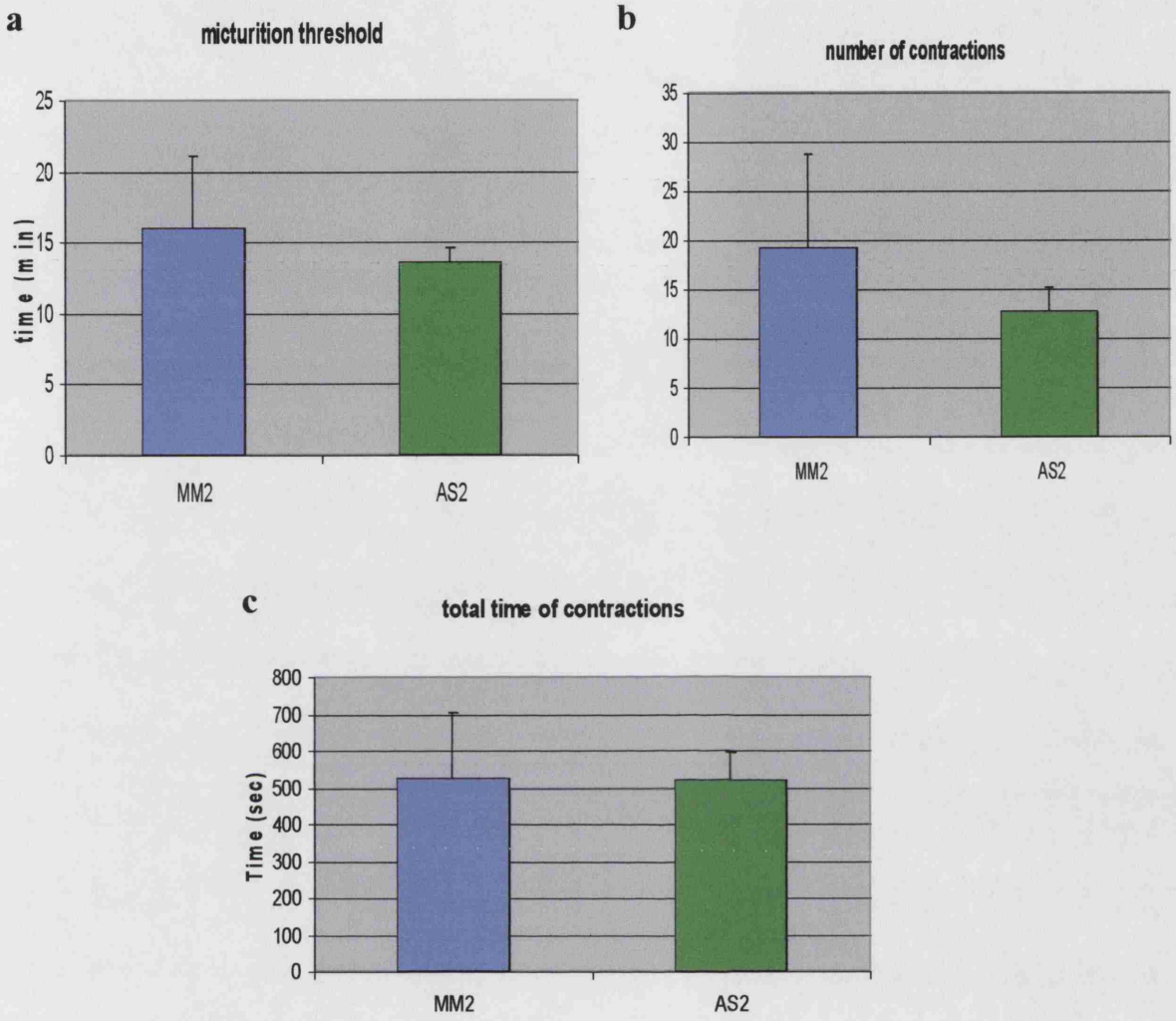


Figure 3.7: No significant difference is observed in the bladder dynamics of the two groups treated with either antisense oligo AS-2 compared to the comtrol mismatch MM-2 oligo.a) the time taken to begin contractions (micturition measure) is the same for both groups; b) the number of contractions exhibited is not significantly different; c) The total time during which contractions are exhibited is also unchanged. Data presented as means ±SE (n = 12).

Figure 3.8 Western blot analysis of $Na_V1.8$ protein levels; effect of NGF

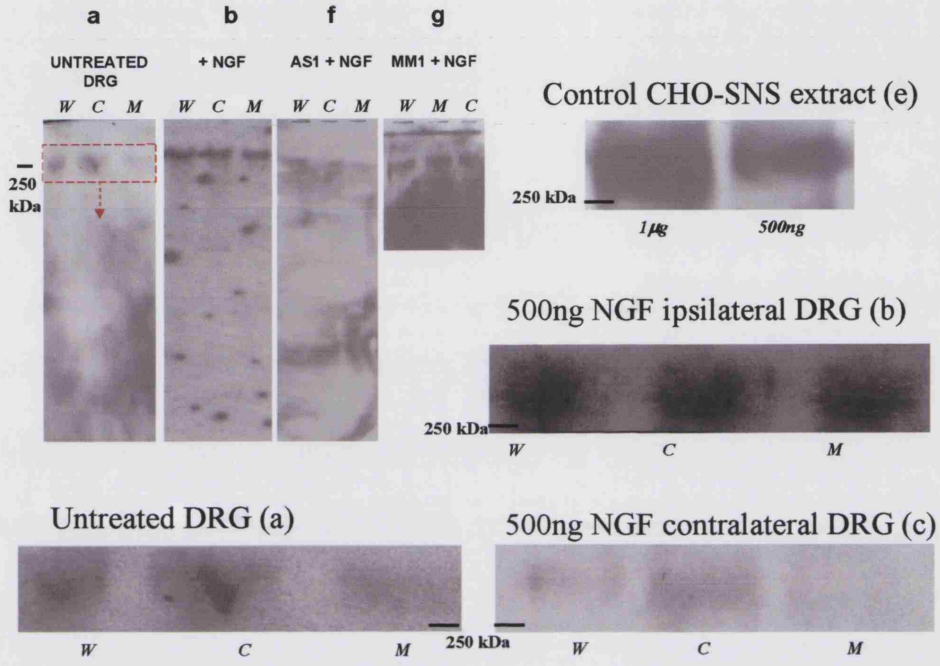


Figure 3.8; Western analysis of DRG proteins extracted from NGF-treated animals. (W - Whole cell protein extract; C - Cytosolic protein fraction; M - Membrane protein fraction). The Control (e) shows CHO-SNS cell whole cell extract loaded at 1 μg/lane or 500ng/lane. All other gel lanes are loaded at 500ng/lane. The panels at the top left demonstrate the single band of $Na_V1.8$ protein at 250kDa. The cell fractions loaded demonstrate that application of NGF (b) increased the quantity of $Na_V1.8$ in all fractions compared to untreated DRG (a). The contralateral DRG (c) remained unchanged after NGF application. Application of AS-1 prior to NGF application reversed this increase in protein (f). This effect was specific as treatment with MM-1 before NGF application did not prevent the upregulation of $Na_V1.8$ protein (g).

Figure 3.9 Effect of AS-1 vs MM-1 infusion on $Na_V1.8$ protein levels

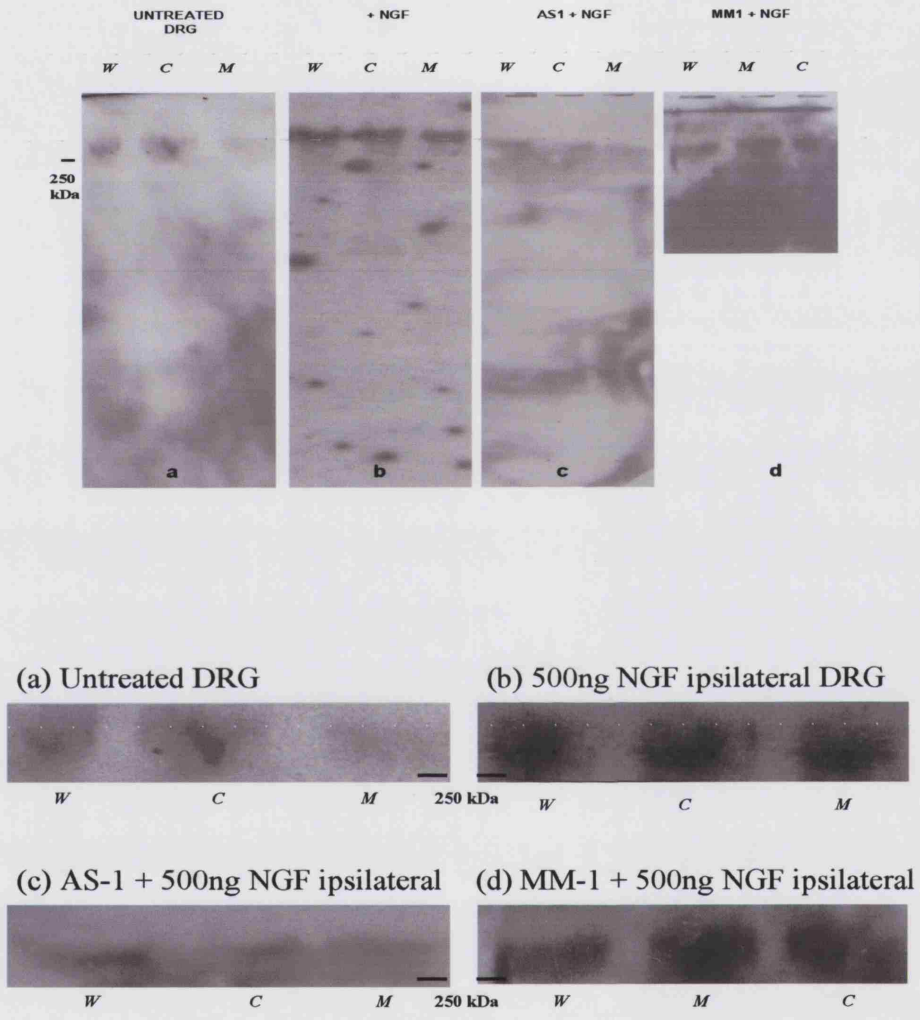


Figure 3.9; Western blots of $Na_V1.8$ protein levels before (a) and after NGF treatment (b), with or without AS-1/MM-1 infusion (W- whole cell fraction, M – membrane fraction, C – cytosolic fraction). The treatment with AS-1 (c) restores the levels of Nav1.8 protein to near normal levels (a) after NGF-mediated inflammation in the ipsilateral paw. This effect is specific to the AS-1 oligonucleotide as MM-1 treatment (d) does not prevent upregulation of $Na_V1.8$ seen upon NGF-mediated inflammation (b).

Figure 3.10 Concentrations of $Na_V1.8$ protein in 500ng protein from various cell fractions taken before and after treatment with NGF (n=6)

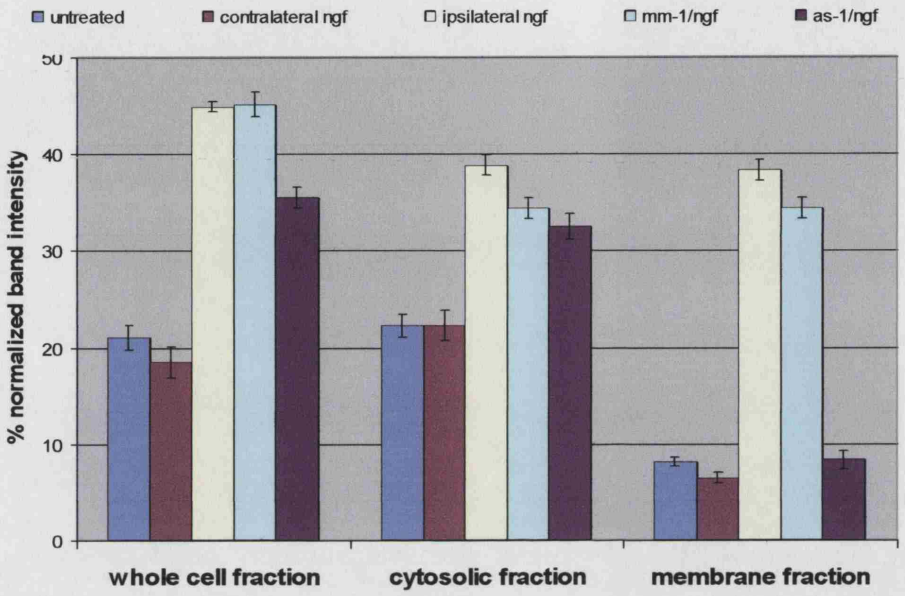


Figure 3.10: $Na_v1.8$ immunoreactivity in DRG cell fractions harvested after intraplantar application of NGF compared to controls receiving no NGF or contralateral DRG extracts. Six readings were taken from each protein band (Figures 3.8 & 3.9) and the mean ± S.E. is plotted. Blue columns show the normalised protein band intensity of whole cell, cytosolic and membrane fraction from untreated control animals. Magenta bars; no effect in the contralateral (to NGF application) DRG protein concentrations. Yellow bars; increase in $Na_V1.8$ after NGF treatment in the ipsilateral DRG. The total level of immunoreactivity has gone up (whole cell control vs. whole cell NGF only) as well as in the cytosolic and membrane fractions. The treatment with MM-1 has no effect on this upregulation in any fraction (green bars). Purple bars; $Na_V1.8$ protein levels in subcellular fractions after NGF inflammation when pretreated with AS-1 antisense oligonucleotides. The total level of protein is increased and is increased in the cytosolic component, but the membrane associated protein has remained at control levels (like untreated DRG and DRG from the contralateral side (of NGF application).

3.6 Discussion

The regulation of the distribution of $Na_v1.8$ protein in DRG cells has been achieved by the knock down of p11 protein expression by means of intrathecal delivery of short non-phosphothiolated oligonucleotides directed against the p11 mRNA sequence. The modulation of the level of $Na_v1.8$ protein found in various subcellular fractions namely the cytosol and the membrane components was shown by Western analysis. These studies showed that the translocation of $Na_v1.8$ protein to the membrane can be reduced by the knock-down of p11 protein.

The behavioural consequences of this manipulation were investigated by analysing the changes to thermal hyperalgesia development in a peripherally applied non-systemic NGF inflammatory model. The data collected shows that p11 is an important component in the *in vivo* shuttling of mature $Na_v1.8$ protein to the membrane. The application of NGF subcutaneously increases the amount of $Na_v1.8$ protein as shown by the level seen in total protein extracts from treated animals compared with non-treated controls. P11 protein then acts to shuttle $Na_v1.8$ to the membrane as shown by the decreased $Na_v1.8$ protein levels in the membrane fraction of P11 antisense treated rats. This analysis was done by means of Western Blots.

The initial phase of hyperalgesia which can be observed in the NGF-induced thermal hyperalgesia model was significantly affected by the lack of $Na_v1.8$ protein in the membrane, suggesting that this acute phase is dependant on the activity of $Na_v1.8$ channels in the normal animal. This phase also requires the participation of mast cell degranulation processes at the injury site, a process in which p11 has not been implicated.

The second phase was seen to be reduced but was not abolished. This suggests that it is a more complex phenomenon, perhaps involving effects mediated through transport of the NGF to the dorsal horn from the peripheral site of application, or the activation of secondary inflammatory processes such as NGF-induced upregulation of glucocorticoids, or arachidonic acid metabolites.

These studies support the earlier work of Okuse *et al* (2002), who showed by means of a yeast two-hybrid screen that p11, is an interactor of $Na_v1.8$. Transfection of p11 into a cell line CHO-SNS22 led to the shuttling of the channel protein to the membrane where it began to mediate a TTX-r sodium current, ascribed to $Na_v1.8$. The co-localization of

$Na_v1.8$ and p11 protein of the plasma membrane leading to functional $Na_v1.8$ channels was also demonstrated.

The loop connecting the two EF hands and the α-helix of the second EF hand has been suggested as important in mediating the interaction between the two proteins (Poon et al., 2004). This interaction may allow p11 to interact with $Na_V1.8$ without the participation of the annexin II heavy chain (P36). Alternatively, P11 may act to translocate the mature $Na_v1.8$ protein in concert with p36, with which it forms a heterotetramer. The C-terminal of p11 interacts with p36 (annexin II heavy chain) (Kube et al., 1992) leaving the molecule free to interact with $Na_v1.8$ on its N-Terminal. Annexin II heavy chain is widely expressed in DRG cells but is particularly abundant in small-diameter neurones ((Naciff et al., 1996).

$Na_v1.8$ plays an important role in pain pathways. $Na_v1.8$ null mice were shown to have important and specific effects in pain perception of NGF-induced thermal hyperalgesia ((Kerr et al., 2001). In particular it was shown that these mice exhibited a partial analgesia to noxious thermal stimuli induced through systemic NGF application, implicating $Na_v1.8$ in the transmission of these signals. The hyperalgesic response 6 hours after NGF treatment was not entirely abolished however, indicating that other non-$Na_v1.8$-dependant pathways are still functional. One important factor in the development of this initial phase of hyperalgesia is mast cell degranulation, however it is not easy to understand how the knock-down of p11 in the DRG cell bodies can interfere in mast cell degranulation in the periphery.

Studies have also shown that inflammatory mediators such as PGE_2 can modulate the TTX-r sodium currents in small diameter neurones, (Gold, 1999; Gold et al., 2002; England et al., 1996b). Reducing the level of $Na_v1.8$ protein by means of antisense oligodeoxynucleotides (ODN) has provided evidence for its importance in the mediation of PGE_2 induced hyperalgesia (Khasar et al., 1998). There is therefore a possibility that p11 may play a role in this pathway as well though interference with the phosphorylation of $Na_V1.8$ (Fitzgerald et al; 1999). There have not been any studies which show the effect of PGE_2 on p11 levels directly *in vivo* however.

Earlier work showed that whilst $Na_v1.8$ mRNA expression is only slightly increased in NGF-induced inflammation, the plasma membrane concentration of mature $Na_v1.8$ channel increases significantly (Okuse et al., 1997). This result has been replicated here as

shown by western analysis of the protein concentrations before and after NGF application *in vivo*. The size of the band observed was a little more that 250 kDa, this compares favourably to the earlier published data i.e. > 220 kDa (Okuse et al., 1997) and to the band size found in human preparations of 260 kDa (Yiangou et al., 2000), this group also demonstrated a 220kDa variant which was not observed in this study on rat tissues.

NGF deprivation was shown to reduce the $Na_v1.8$ expression along with TTX-r sodium currents in IB4-negative DRG neurones *in vivo* (Fjell et al., 1999). This effect may be understood more fully if is considered that NGF induces the upregulation of p11 in neuronal cells *in vitro* (Okuse et al., 2002). The apparent affect of NGF deprivation can therefore be seen as a mirror of the reduction in the p11 protein in the cell and its availability to mediate the inflammatory hyperalgesia through translocation of the protein to the plasma membrane (possibly with the participation of annexin II heavy chain molecules).

It is important to examine the data produced by Okuse *et. al.* (1997) where NGF stimulation led to an increase in the membrane bound concentration of $Na_v1.8$ of approximately 25% which was not reflected in the mRNA concentrations after exposure to NGF either *in vitro* or *in vivo*. Similar results have been reported for other voltage-gated channels *in vitro* (Sharma et al., 1993). The location of the *de novo* channels induced upon NGF stimulation has been shown in this study to be not only membrane associated but also located cytosolically. This demonstrates that there may be an upregulation of $Na_v1.8$ upon NGF stimulation and therefore a post-translational effect must be operating to increase the production of the channels if the mRNA levels remain constant. Perhaps the increase in p11 molecules as a result of NGF stimulation help to increase the maturation of the $Na_v1.8$ polypeptide by more efficiently shuttling completed chains away from the RNA strand and toward their final membrane location allowing an increased rate of translation. This would imply that there is an interaction between p11 and the protein translation machinery directly or indirectly. Such interactions have yet to be demonstrated however.

Many cutaneous experimental inflammatory models such as carrageenin-induced or Complete Freund's Adjuvant (CFA) induced inflammation is thought to act at least partially though the activity of NGF, implicating further p11 in the inflammatory response though mediation of nascient $Na_v1.8$ molecules.

In the bladder there is as yet only circumstantial evidence that NGF may be important. However large doses (order of 1mg/Kg) have been shown to produce hyperexcitibility similar to that observed with turpentine-induced bladder inflammation model (Dmitrieva et al., 1997). Other work has suggested that an inflammation of the bladder can lead to a referred thermal hyperalgesia in the hind paws which is NGF dependant (Jaggar et al., 1999), and that $Na_v1.8$ is robustly expressed in the bladder afferents (Black et al., 2003). $Na_v1.8$ is also involved in mediating visceral pain in the rat (Yoshimura et al., 2001b; Yoshimura et al., 2001a). Investigations were therefore undertaken to determine if the cerebrospinal application of p11 antisense molecules could affect the baseline levels of bladder activity.

There was no statistically significant effect to be seen in any of the readings taken for micturition time; number of super-threshold contractions or total time of contractions. This suggests that the $Na_v1.8$ channels present in the bladder afferents originating in the DRG do not require the activity of p11 to maintain function or perhaps are reliant on alternative shuttling mechanisms or express p11 at levels too high to be effectively reduced with an antisense protocol. However, it cannot been ruled out that p11 may play a part in mediating the TTX-r current increase in bladder afferents seen on inflammation of the bladder, in a similar way that it does in the other DRG afferents terminating in the periphery (sensory neurones). This can be tested by repeating the antisense experiment but using a turpentine–induced bladder inflammation model. It is of note that the knock-down of p11 by antisense molecule AS-1 in this study also had no significant effect on the resting levels of thermal nociceptive levels in the paw, as assessed by the Hargreaves test. This suggests that p11 may not be important in maintaining the base level of thermal sensitivity and $Na_v1.8$ may therefore be implicated only under active inflammatory stimulus.

The fact that p11 is important in translocation of the $Na_v1.8$ protein to the membrane but no change was seen in the baseline levels of response to thermal stimulus suggests that the physiological (non-inflamed) transmission of a noxious heat stimulus does not rely on a large number of functional $Na_v1.8$ channels or acts in concert with other sodium channels as well. This contrasts with the signal transmission in the NGF-induced inflammatory state. Here the up-regulation of $Na_v1.8$ channels and their increased density on the membrane may contribute to the reduction of the threshold for the transmission of a

stimulus, as reflected in the lowered response times. It is possible that only in the case of inflammation where there are a large number of $Na_v1.8$ moieties to be shuttled to the membrane that the number of p11 molecules becomes a limiting factor. The reduction of p11 molecules through this method of antisense oligonucleotide introduction by canulation of the spinal cord does not achieve the knock-down efficiency required to affect the density of channels in the membrane to such an extent that the transmission of non-nociceptive impulses is impaired. P11 is abundantly expressed in many tissues as well as in small diameter nociceptors, however the level of p11 knock down achieved was not assessed due to the unavailability of a suitable p11 antibody. The transfection efficiency of directly applied ODN's was assessed by using Texas-red labelled ODN's; it was found that 73.3 ± 2.9% of small diameter cells (< 35μm) were labelled and 80.1 ± 4.6% of large diameter cells (> 35μm) were labelled (n=5). This gives an overall transfection efficiency of 74.2 ± 2.5% (data presented and means ± SEM, n=5).

Tissue damage which accompanies inflammation may also involve the action of p11 in some way. Nitric oxide (NO) is another inflammatory mediator released from damaged cells, reviewed in (Kidd and Urban, 2001). P11 protein and mRNA has been shown to be up-regulated in the presence of NO, at least in non-neuronal cells (Pawliczak et al., 2001). It is therefore possible that the antisense molecules are also interfering with and causing the degradation of *de novo* p11 proteins induced by NO release at the site of inflammation. This reduction in p11 is acting to further depress the insertion of $Na_v1.8$ channels in the membrane which accompanies the inflammatory response and resulted in a much less severe thermal hyperalgesia in the antisense treated animals compared with control animals.

There was no statistically significant effect upon the baseline levels of thermal sensitivity in any of the groups tested. This suggests that either a) $Na_v1.8$ plays a diminished role in determination of resting levels of thermal nociception and therefore the reduction in *de novo* insertions to the plasma membrane and/or the stabilization of channels already in the membrane by p11/p36 complex upon p11 protein reduction, does not lead to a change in the mechanics of the baseline responses, or b) p11 is only a limiting factor in the insertion of de novo channels under inflammatory conditions, the normal abundance of p11 being sufficient to traffic the uninflamed channel insertion rate and its down-regulation does not affect those already present in the membrane directly

thus p11 protein reduction becomes important only in increased channel synthesis conditions. This question cannot be adequately addressed by the data so far; the knockdown of p11 was not directly investigated owing to the lack of a suitable antibody. The extent of p11 reduction cannot be checked leaving open the possibility that only a small fraction of this widely and abundantly expressed protein was affected by the treatment.

P11 has recently been shown to interact with two other classes of membrane-located ion channels, the potassium channel TASK-1 (Girard et al., 2002) and the calcium-regulating channels TRPV5/6 (Van den Graaf et al., 2003). The reduction of available p11 molecules in these experiments may have affected the localization and/or functioning of these channels as well. The conclusions of these studies must therefore be evaluated according to the likely roles of these two classes of channels in cell excitability, in the models assessed, before a conclusive answer of the requirement for membrane-bound $Na_V1.8$ in the establishment of inflammatory pain behaviour can be reached.

Chapter 4: Studies on a Cre-recombinase expressing mouse strain to determine the level of expression achieved with a promoter derived from the Ncx/Hox11L.1 sequence.

4.1 Synopsis

The expression pattern of an Ncx/Hox11L.1 Cre-expressing mouse strain was investigated to determine its suitability in effecting specific ablation in genes in defined subsets of neurones in dorsal root ganglia (DRG). In particular, whether nociceptive subpopulations of neurones would efficiently express Cre recombinase under the control of the Ncx promoter region was investigated. Such an outcome would allow genes implicated in nociception and pain pathways to be studied more precisely. The crossing of the Ncx-Cre expressing strain with another in which a gene of interest has been floxed (allowing its recognition and deletion by the enzyme) will result in the specific ablation of this target sequence in the cells expressing Cre-recombinase only. This work therefore identifies the population of cells in which this event will occur.

The promoter sequence derived from the Ncx homeobox gene was used to drive Cre-expression in an attempt to limit the expression pattern to cell populations which express the Ncx gene *in vivo*. The Ncx gene was chosen because it has been found to direct expression in a wide variety of neural crest-derived tissue including DRG (Hatano et al., 1997b).

The use of conditional deletion events mediated by Cre-recombinase under the control of tisse specific promoters (Tsien et al., 1996) was adopted in an attempt to alleviate or eliminate confounding effects due to developmental deficiencies and/or lethality which can accompany unrestricted Cre-mediated gene deletion with strong promoters such as CMV (cytomegalovirus promoter) (Orban et al., 1992). The use of these strong promoters in fact mimics the situation encountered with unconditional knock-out animals when delivered unselectively, and their use depends on the availability of other secondary selection methods to further control the expression of the Cre enzyme.

In order to define the adult expression pattern of the Ncx-Cre mouse strain it was crossed to a reporter strain Rosa-26R (Soriano, 1999) (which expresses functional β-galactosidase when exposed to Cre-recombinase) and a wide variety of tissues including

brain, spinal cord, DRG and other sympathetic ganglia were assessed for the presence of Cre-activity as denoted by the presence of active β-galactosidase. Population of neurones can be therefore be defined by this strain. This will therefore allow it use in ablating genes of interest specifically in a defined population of sensory neurones.

The development of other Cre-expressing mouse strains with defined tissue expression in complementary neuronal subpopulations will aid in the elucidation of putative nociceptive genes. A series of crosses with specified conditional Cre-expressing lines can then be made, each one having the floxed gene of interest deleted in a defined subpopulation (Wagner et al., 2001). These complementary crosses can then be exposed to the same behavioural tests to determine the particular role of the gene of interest in these subpopulations (Crawley and Paylor, 1997).

In this manner the activity of a particular gene in nociception can be defined. This may illuminate the importance of this pathway and highlight it as a possible site of invervention for analgesic research.

4.2 Introduction

Gene targeting in embryonic stem cells (ES cells) and mice has been used to study the *in vivo* functions of genes during development and adult life. Although the standard approach has been informative, complications due to lethality at the embryonic stage or developmental problems has obscured the role of many genes, precluding their assessment with standard protocols (Joyner, 1994).

The creation of delimited Cre-expression animal strains has been an important development in recent years. Cre mediates the excision of sequences flanked by loxP sites therefore controlling the expression of Cre-recombinase allows the excision event to be precisely controlled at both a temporal and tissue level(Orban et al., 1992).

Two strains of Cre-expressing mice are to be investigated in this thesis (chapter 4 & 5) to determine which allows the best expression of the Cre-recombinase enzyme in nociceptors. The best strain can then be used to specifically ablate putative sequences involved in pain pathways specifically in nociceptors allowing a more precise characterisation of their involvement in nociception. This chapter describes experiments carried out to determine the expression pattern and hence usefulness of an Ncx-Cre expressing strain.

The use of conditional gene manipulation methods provides several advantages over conventional gene targeting approaches. The success of conditional gene modification strategies depends on the controlled expression of the Cre-recombinase enzyme in a defined spatial (tissue specific) or temporal pattern (stage specific).

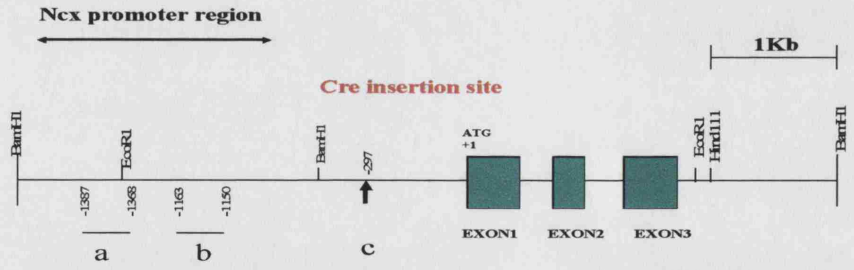
Spatial control of proteins relies on the identification and use of gene control sequences (promoter, enhancer sequences) which are limited in their utilization to the tissue under investigation (Tsien et al., 1996). Temporal control is generally determined by the developmental regulation of the promoter chosen to drive the Cre-recombinase gene, or through the use of a ligand such as tetracycline to induce the expression of the Cre-gene (St Onge et al., 1996). Both gain-of-function as well as loss-of function mutations can be created using this system (Metzger and Chambon, 2001).

The understanding of nociceptor specific transcription determinants is important for the targeted study of genes in pain pathways. Many genes implicated in nociception are also required during neurogenesis e.g. NGF, BDNF (Lewin and Barde, 1996), and/or

non-nociceptive functions, such as the annexin subunit p11 (Harder and Gerke, 1994). As a result, total gene ablation of these sequences can lead to animals that are either non-viable or exhibit abnormal physiology complicating the interpretation of experimental results (Joyner, 1994).

The application of the lineage/cell type-specific knock out methodology has been successfully used to create transgenic animals which allow a more precise examination of systems to be achieved. Tsien et al (1996) have been successful in the creation of a mouse strain in which the expression of the Cre-recombinase is limited to CA1 pyramidal cells located in the hippocampus. They achieved this by driving the Cre-recombinase expression under the control of the αCaMKII promoter. Fortuitously they also found that the expression pattern was stage-specific, with the Cre expression occurring after the third postnatal week. They suggested that this time was sufficient to avoid most if not all developmental effects of the gene deletion, thus allowing more faithful explanation of the native gene function in adulthood (Tsien et al., 1996).

4.2.1 The Ncx/Hox11L.1 gene



a - Tissue specific expression element
b – Retinoic acid response element
c – Transcription initiation site

Figure 4.1 Ncx gene structure

Figure 4.1: Structure of the Nxc gene and promoter region showing the Cre-recombinase insertion site after the native Ncx promoter region (Hatano et al., 1997b).

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4.2.1 The Ncx/Hox11L.1 gene

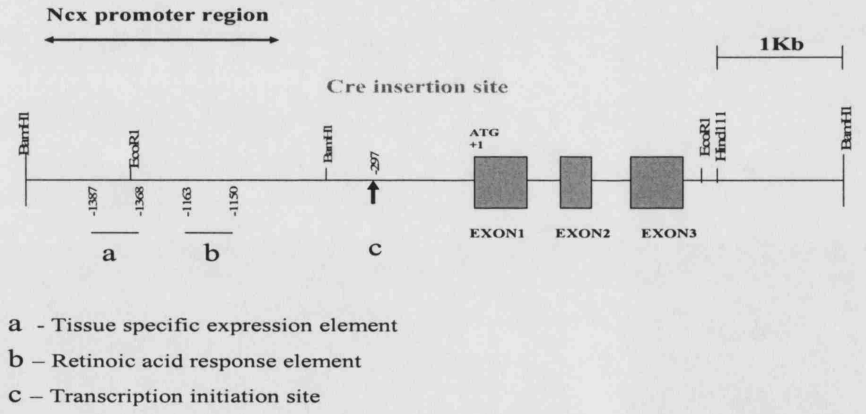


Figure 4.1 Ncx gene structure

Figure 4.1: Structure of the Nxc gene and promoter region showing the Cre-recombinase insertion site after the native Ncx promoter region (Hatano et al., 1997b).

The murine Ncx/Hox11L.1 gene (Figure 4.1), belongs to a Hox11 homeobox gene family which is located apart from the four main clusters of homeobox genes in the mouse and human genomes (Wen et al., 1994).

The Hox11 family which includes Ncx/Hox11L.1 as well as others, have been identified by probing the genome with sequences derived from Hox11 itself and includes the mouse gene tlx-1 located on chromosome 14 (Raju et al., 1993), and a DNA-binding nuclear transcription factor (DRG11) which has been found to have a limited expression pattern restricted mainly to nociceptive sensory neurones and their central target area the dorsal horn (Dear et al., 1993; Chen et al., 2001).

This transcription factor (DRG11) has been investigated recently through studies of DRG11 knock-out mice. These animals illustrated that DRG11 plays a major role in the survival of nociceptive neurones and their proper innervation into targets located in the dorsal horn. The deletion of DRG11 lead to animals with severely reduced nociceptive responses(Chen et al., 2001). This result suggested that other members of the Hox11 gene family including Ncx may also be similarly restricted in expression and function.

4.2.2 Ncx expression

Ncx is expressed in a subset of neural crest derived tissues such as dorsal root ganglia (DRG), cranial nerve ganglia, sympathetic ganglia, and enteric nerve ganglia in embryos between days E9.5 and E13.5 (Hatano et al., 1997b). Nociceptive neurones are also derived form neural crest cells, as are the majority of sensory neurones despite their high degree of specialisation). (Hatano et al., 1997b; Iitsuka et al., 1999; Anderson, 1993; Stemple and Anderson, 1993).

In the adult mouse Ncx expression was found in the enteric nerve ganglia and adrenal glands (Hatano et al., 1997a). However, whilst Ncx expression has been recorded in embryonic mouse DRG and sympathetic ganglia, data on the expression pattern, specifically which cell types express the protein or the level of expression in terminally differentiated tissue, is not currently available in the literature. This chapter presents data of the expression of functional Cre recombinase when driven by the NCX-promoter in a wide variety of neuronal tissue of adult mice.

4.2.3 Ncx knock-out mice

The ablation of the Ncx gene in mice was undertaken by Hatano et al. (1997) by the replacement of a 0.9-kb region containing the 5' flanking region and a part of the first exon including the start ATG with the neo resistant cassette. Homozygous Ncx (-/-) mice were born at the expected Mendelian frequencies showing that the deletion did not lead to any lethal congenital defects. The mice appeared normal but at 5 weeks post-partum approximately 50% of the animals died due to megacolon. This resulted from a hyper-innervation of enteric neurones in the proximal colon.

4.2.4 Ncx-Cre mouse development and investigation

The suitability of an Ncx-controlled Cre-recombinase expressing mouse line in the study of pain pathways in the peripheral nervous system remained unclear however. By using the Ncx (neural crest homeobox)/Hox11L.1 promoter to drive Cre expression, it is hoped that the enzyme will mimic the temporal and spatial expression pattern identified for Ncx itself (Hatano et al., 1997b), including expression in sensory neurones including the nociceptors.

Specifically, if the expression of Cre-recombinase under the control of the regulatory element of the Ncx/Hox11L.1 gene was restricted to a sub-population of DRG neurones, this would allow the selective ablation of genes in these neurones. This strain could then be used to elucidate pain pathways in the peripheral nervous system whilst avoiding effects due to large scale deletion of the genes under investigation providing penetration of the Ncx gene expression in the target population was good.

The engineering of a Cre-expressing mouse line under the control of the Ncx promoter was achieved (Jessell et al. personal correspondence). This group inserted the functional Cre-recombinase coding sequence into the Ncx gene at the transcription initiation site, 267 amino-acids upstream of the first coding exon. This allowed the functional expression of Cre from embryonic stage 9.

We then crossed Ncx-Cre expressing mice were crossed with a reporter stain in order to determine the expression pattern in adult animals. Homozygous Ncx-Cre mice were crossed with homozygous ROSA-26 reporter mice to obtain double heterozygous

Ncx-Cre/Rosa-26 strain (F116) which were used for the expression studies. The use of heterozygous animals for the Ncx-Cre allele would avoid the effects noted in the Ncx-knock-out mice cited above.

4.2.5 ROSA-26 reporter strain

The ROSA-26 reporter strain (Soriano, 1999; Mao et al., 1999) was created by modifying the β-galactosidase neomycin phosphotransferase fusion gene (βgeo)-trapped ROSA26 locus such that the enzyme is only transcribed after Cre-mediated excision of lox-P flanked sequences.

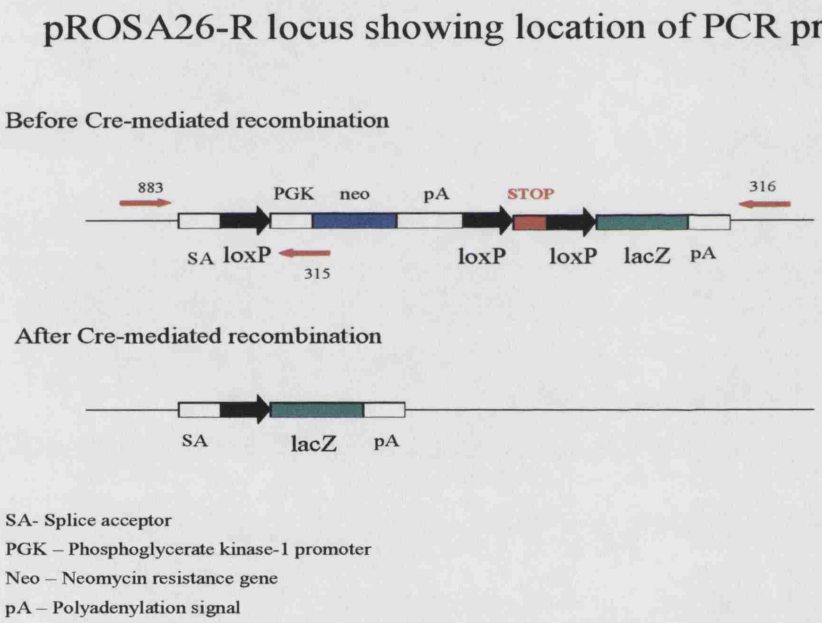


Figure 4.2 pROSA-26R reporter strain locus

Figure 4.2: Rosa-26 reporter strain locus before and after a Cre-mediated excision event illustrating the excision of the STOP (polyadenylation) signal which prevents the expression of the β-galactosidase gene in the naïve gene construct. The primers used to indicate the presence of the transgene are indicated in red.

The ROSA26 locus was chosen as the site for this intervention because it was identified as a ubiquitously expressed locus during embryonic development (Friedrich and

Soriano, 1991; Zambrowicz et al., 1997). The expression of the LacZ gene is therefore turned on when the Cre-enzyme is made available (through expression using a promoter which allows temporal and/or spatial control) leading to the deletion of the stop signal and transcription of the β-galactosidase gene (Figure 2.2).

The β-galactosidase gene presence can be subsequently detected by a number of substrates, all of which have galactose linked through a β-D-glycosidic bond to a moiety whose properties change upon liberation from galactose (Wallenfels and Weil, 1972). The most common substrate is an indole derivative which turns blue upon cleavage, 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal) (Holt and Sadler, 1958).

The presence of functional β-galactosidase in the Rosa-26R strain indicated that at some point before the assay the gene for β-galactosidase has had its LoxP sites removed by the action of Cre-recombinase allowing it to be functionally transcribed. The crossing of a Cre-expressing strain with the Rosa-26R reporter strain is therefore an accurate method of determining the activity of Cre-recombinase through the presence of β-galactosidase transcription in a cell.

4.3 Objectives

The objective of this study was to determine the adult expression pattern of Cre-recombinase in a transgenic mouse engineered to express the enzyme under the control of the Ncx/Hox11L.1 promoter through the measurement of functional β-galactosidase in an Ncx-Cre x Rosa 26R cross. Specifically, the expression obtained in peripheral nervous tissue, including dorsal root ganglia (DRG), superior cervical ganglia (SCG), trigeminal ganglia, and enteric ganglia as well as the expression in adult brain will be determined.

This study was undertaken to identify possible uses for this Cre-expressing transgenic mouse strain in the study of genes involved in pain pathways in the peripheral nervous system.

Limitations in the use of transgenic technology centres on the high cost of these protocols both in terms of time and monetarily. It was hoped that the use of alternative genetic manipulation methods using Cre-expressing HSV derived vectors (Chapter 2) and antisense downregulation approaches (Chapter 3) could be used. However investigations into both of these approaches have highlighted drawbacks preventing their use in studying

specific sensory neuron subpopulations in the PNS. The investigation of these two transgenic Cre-expressing lines (chapter 4 & 5) is therefore undertaken as they represent the best way of achieving these aims at present in defined subpopulations of DRG neurones.

4.4 Materials and methods

4.4.1 Genomic DNA Preparation

Tissue was collected from 0.5cm lengths taken from the tip of the tail, and placed in 700µl Tail buffer (50mM Tris-HCL, pH8.0; 100mM EDTA; 100mM NaCl; 1% SDS) containing 0.5mg/ml proteinase K (20µl) (Sigma-Aldrich, Ca.). This preparation was incubated overnight at 37 ^{O}C to break-down the tissue allowing the genomic DNA to be more easily harvested. 250µl of saturated NaCl (6M in dH_2O) was added to the resultant slurry and mixed by inversion to precipitate the proteins. The mix was centrifuged (14K rpm, 10 min, RT) and 750µl of resultant supernatant transferred to a new Eppendorf tube. 750 µl isopropanol was added to precipitate the DNA and again centrifuged to collect the precipitate. The resulting DNA pellet was washed in 70% ethanol, air dried and resuspended in 30µl of dH_20. DNA samples were kept at -80 ^{O}C to prevent degradation

4.4.2 Genomic analysis of mice

The genomic DNA harvested from the test animals was subjected to PCR-based protocols to determine the genotype.

4.4.2.1 ROSA-26R gene detection

To determine whether the floxed β-galactosidase-neomycin phospho-transferase fusion gene was inserted into one or both alleles at the ROSA26 locus a set of primers were used (supplier; Sigma-Genosys Ltd, Pampisford, UK) which identified the presence of the lacZ gene at the locus using a PCR reaction to identify the transgene. The protocol used was developed by B. Zambrowicz and P.Sorriano (Soriano, 1999). This protocol allows the differentiation of the wild-type allele (550bp) from the transgene (250bp). The location of the primers used is indicated on Figure 4.2 above. Note that the primer designated 316 is located downstream of the inserted lacZ cassette and as such is too far to be efficiently amplified when the βgeo construct is properly inserted and therefore the wild-type band (550bp) is only seen in the wild type gene (Soriano, 1999) (Diagram 4.2).

Forward PCR Primer 813 5' GCG AAG AGT TTG TCC TCA ACC 3'

Reverse PCR Primer 315 5' GGA GCG GGA GAA ATG GAT ATG 3'

Reverse PCR Primer 316 5' AAA GTC GCT CTG AGT TGT TAT 3'

PCR reaction mix for one reaction (total 30µl) as follows; Taq polymerase (0.6µl); 10x PCR Buffer (Gibco, UK) (3µl); 25mM $MgCl_2$ (1.6µl); 5mM dNTP's (0.6µl) (1:1:1:1 mix of dATP, dGTP, dTTP, dCTP from Sigma, UK); 20µm Forward primer (1µl); 20µm Reverse primer 316 (1µl); 20µm Reverse primer 316 (1µl); dH_20 (20.2µl); Sample DNA (1µl).

PCR Cycling conditions – PCT-220 DNA Dyad (MJ Research) Machine

1 cycle	1 x 94^{O}C	1 min
40 cycles	1 x 94^{O}C	1 min
	1 x 63^{O}C	30 s
	1 x 72^{O}C	2 min
1 cycle	1 x 72^{O}C	10 min

The resultant products were resolved on a 1.5% agarose (Invitrogen) gel made up in 1 x TAE buffer and photographed.

4.4.2.2 Ncx-Cre gene detection

In order to determine if the Ncx-Cre construct was inherited, a PCR based protocol was used to check for the presence of the Cre sequence utilizing primers designed to anneal within the Cre sequence itself. This primer pair was designed to anneal to the regions between base-pairs 35 – 58 (Cre Seq2s) and base-pairs 218 – 255 (Cre Seq5a) of the Cre sequence allowing them to be used to detect the presence of Cre in the transgenic mouse as well as in the original plasmid. A positive result was indicated by the presence of a 220bp band only (C. Stirling personal correspondence).

Forward Primer Cre Seq2s 5' CTG CAT TAC CGG TCG ATG CAA CGA 3'

Reverse Primer Cre Seq5a 5' AAA TGT TGC TGG ATA GTT TTT ACT GCC 3'

PCR reaction mix for one reaction (total 30µl) as follows; Taq polymerase (0.6µl); 10x PCR Buffer (Gibco, UK) (3µl); 25mM $MgCl_2$ (1.6µl); 5mM dNTP's (0.6µl) (1:1:1:1 mix of dATP, dGTP, dTTP, dCTP from Sigma, UK); 20µm Forward primer (1µl); 20µm Reverse primer (1µl); dH_20 (21.2µl); Sample DNA (1µl).

PCR Cycling conditions – PCT-220 DNA Dyad (MJ Research) Machine

1 cycle	1 x 94°C	2 min
40 cycles	1 x 94°C	30 s
	1 x 60°C	30 s
	1 x 72°C	1 min
1 cycle	1 x 72°C	10 min

The resultant products were resolved on a 1.5% agarose (Invitrogen) gel made up in 1 x TAE buffer and photographed.

4.4.3 Tissue collection and sectioning

Adult double heterozygous (ROSA-26R/Ncx-Cre) animals were transcardially perfused with 4% ice-cold PFA (4% paraformaldehyde in 1 X PBS, pH 7.4) and L4/5 dorsal root ganglia (DRG), trigeminal ganglia, superior cervical ganglia (SCG), enteric wall samples from ileum, brain and lumbar region spinal cord dissected and post-fixed for 1hour in ice-cold 4% PFA solution. The tissues were then cryoprotected in 30% sucrose solution in 1 X PBS with 0.02% sodium azide added to aid preservation.

The DRG, SCG, trigeminal ganglia and ileum segments were embedded in OCT compound (Sigma, UK) and cut at 10µm thickness sections using a cryostat and directly thaw-mounted on to gelatine coated slides. These sections were taken serially with every tenth one on the same slide.

Outer layers of the ileum were also stripped with a cotton bud to produce longitudinal layer slices to detect the myenteric plexii (Figure 4.3). These sections were then floated onto slides for staining.

The cryoprotected brain and spinal cord were sectioned at 14μm on a freezing microtome using a 5% sucrose solution (in 1 X PBS) to attach the tissue to the block. These sections were then float-mounted on to slides.

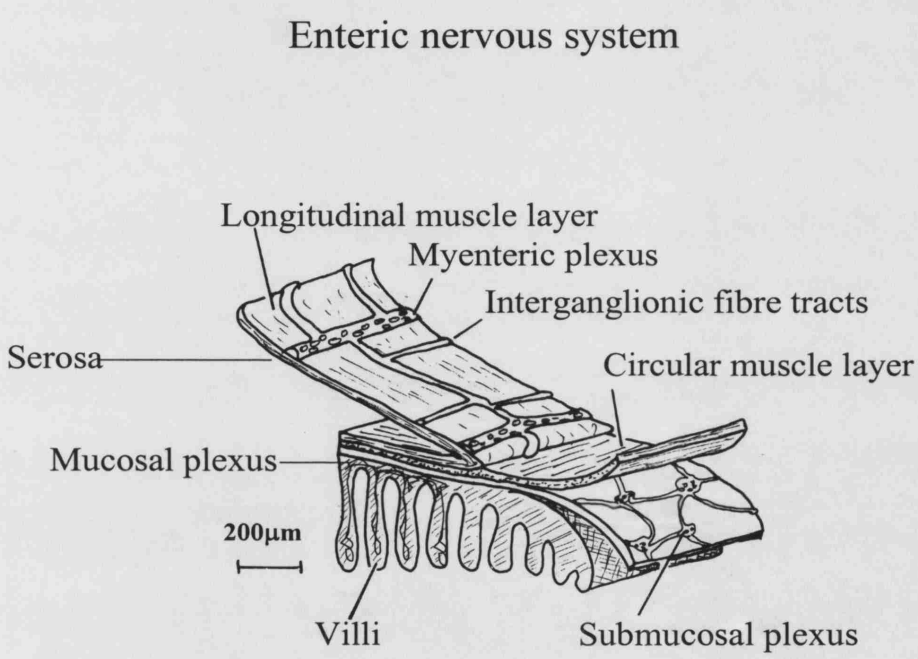


Figure 4.3 Anatomy of enteric nervous system

All slides were air dried for one hour and then stored at -80^{O}C until use with desiccant to control the humidity and prevent degradation of the material.

4.4.4 Immunohistochemistry

10μm sections were immunolabelled with the following antibodies using a double labelling protocol. Detection of large diameter neurones was achieved by applying anti-N52 monoclonal primary antibody (1; 1000) (NF200 clone N52 mouse monoclonal IgG Sigma (UK)) visualised with Mouse FITC conjugated secondary antibody (1; 500). Detection of the small diameter neurones; primary, rabbit polyclonal anti-peripherin (1;

1000) (Chemicon, CA.) visualised with anti-rabbit IgG conjugated Rhodamine red (1; 500).

4.4.4.1 Double-labelling of histological sections

1) Slides washed in 3 X 10 minutes 0.1M PBS
2) Blocked with 10% goat serum in 0.1M PBS (solution 1); 2hrs RT
3) Primary antibody mix in Solution 1
    i. Monoclonal αN52 (1:1000)
    ii. Polyclonal α peripherin (1:1000)
4) Incubate overnight at $4^{O}C$
5) Slides washed in 3 X 10 minutes 0.1M PBS
6) Secondary fluorescent antibody mix in Solution 1
    i. goat α rabbit Rhodamine red (1:500)
    ii. a-mouse FIT-C green (1;500)
7) Incubate 2 hrs at room temperature.
8) Slides washed in 3 X 10 minutes 0.1M PBS
9) Slides mounted with Citifluor (50:50 glycerol/PBS mixture)

4.4.5 Detection of β-galactosidase activity

An X-gal based protocol was used to indicate the presence of functional β-galactosidase enzyme which indicates that a recombination event between the LoxP sites mediated by Cre-recombiase has occurred removing the stop signal from the β-galactosidase sequence allowing its transcription to occur. The slides were placed in the stain solution overnight at 37 ^{O}C to detect functional β-galactosidase enzyme presence.

<u>Stain solution made up in 1 x PBS (pH7.4)</u>

100mM Potassium Ferricyanide
100mM Potassium Ferrocyanide
0.1% (V/V) Nonidet-P40

0.2% (V/V) Sodium Deoxycholate

100mM $MgCl_2$

2.5% 40mg/ml X-gal substrate in DMSO

After immersion in the stain solution, the sections were then cleared in 1 X PBS and counterstained with neutral red to highlight negative cells and mounted using Gelmount (Invitrogen, UK).

4.4.6 Neutral red stain

1) 2 x 1min dH_20
2) 1 x 2 min neutral red solution (1g/100 ml Acetate buffer pH4.8)
3) 1 x 1min dH20
4) 2 x 70% ethanol
5) 2 x 1 min 100% ethanol
6) 2 x 1 min Histoclear
7) Mount slides

4.4.7 Counting of neurones

Sections were observed using a Leica Transmitting Microscope (Leitz DMRB, Leica GmbH. Germany) under visible light, and phase 1 contrast to detect the Blue X-gal positive cells from the background neutral-red stained cells. Epifluorescence with appropriate filter blocks were used to observe the secondary fluorescent labels (Rhodopsin red and FITC-green). The images were digitally captured, one for each fluorophore plus one visible to detect the X-gal staining pattern using the Xilix microimager in conjunction with Openlab 2.0 software. Images were then placed in Photoshop 5.0 for counting.

The accuracy of the estimate (e) of a particular cell population compared to the actual value (E) depends on the sample size. As the sample size increases so does the accuracy of the measurement. In order to obtain a reproducible estimate of the percentage of neurones expressing Cre in the Ncx-Cre expressing mouse tissue, approximately 1,000 neuronal profiles were assessed for sympathetic ganglia and between 1,500-2,000 neurones in the case of DRG. These represent between one quarter and one third of the

total population of cells (as 28-30% of the whole sections harvested per tissue were counted in their entirety with the method outlined below).

The sample population was drawn from sections which were 90μm apart along the entire length of the DRG. This was done to eliminate errors due to clustering of particular subpopulations to particular regions of the DRG superstructure.

The total number of neurones was obtained by counterstaining the X-gal stained sections with neutral red (T). The number of Cre-expressing neurones was determined by counting positive cell profiles (Blue) on the same section (B).

The determination of Ncx-Cre expression in the ganglia was assessed by counting the total number of β-galactosidase positive neuronal profiles and expressed as a percentage (P) of the total neurones (P = B / T %)

A further count was then done by double staining the subsequent sections in the series with anti-peripherin and neurofilament N-52 antibodies. Visualisation with antibodies directed against characteristic epitopes allowed the total neuron count to be subdivided into large and small neuron type bins.

The percentage of Cre-expressing (β-galactosidase positive) cells could then be split into two subpopulations by comparison with the previous or following section in the series to determine co-expression of peripherin or neurofilament.

Total Cre-expressing cell % P = p(peripherin positive) + p(N52 positive) + p(none)

Three complete slides per series (DRG) were counted in this way and the results combined to obtain a larger sample size per DRG. A population size (n) of between 4-6 DRG were drawn from a minimum of four animals and the resultant percentages were collated to remove effects due to sex and/or weight. The expression pattern of Ncx-Cre is given as percentage positive cells per ganglia, subdivided into peripherin-positive and N52-positive fractions

In order to determine if a representative sample was obtained using this method, an estimate of the actual percentages were obtained by counting one lumbar DRG (L1/2) in its entirety. To do this every section was counted using the 3-dimensional reconstruction of serial sections method where each section the "reference" is compared to the previous and following ones, the "lookup" sections and counting before progressing one section

down the series and repeating the procedure. This technique mimics the Z-Slicing method proposed by Williams and Rakic which contains several advantages over other methods used in the literature by reducing the number of variables to be estimated (Williams and Rakic, 1998).

To determine the number of neurones which were to be counted in a particular reference slice, all neuronal profiles which clearly showed a nucleus were counted, with all others ignored. In the rare case where a nucleus was distributed equally between two adjacent sections, the lookup sections provided the ability to identify these neurones and avoid double counting. This method also eliminated overestimation errors accrued by cells larger that the sectioning distance (10μm) and underestimation errors suffered by cells small than the section height (Guillery and August, 2002).

To enable this count to be carried out efficiently, the data was counted in Photoshop and a check-mark placed on a mask layer to indicate that a particular neuron had been counted in the first section of the series. This mask was then carried forward to the next section in the series to enable the cells which had been already counted to be easily determined by lining up to known visual cues, such as the outline of the DRG before proceeding. In this was an accurate and reproducible determination of the total number of cells could be obtained and hence the percentages.

The DRG which was subjected to this counting protocol was double stained with both α-peripherin and α-N52 antibodies so that the population could additionally be split into small and large diameter neurones populations respectively. Three further slides from the same series were used for this count and the totals obtained were then compared to the result obtained using the sampling protocol to determine any correction value required.

In order to determine the efficiency of sampling three out of every ten sections as proposed earlier, the same lumbar DRG which was counted by the 3D reconstruction method was recounted using the proposed method and the difference assessed to determine the accuracy of the percentages obtained by this protocol and the correction factors required to be applied to the outcomes generated (Table 4.1).

The same sampling procedure (i.e. three out of every ten serial 10μm sections) was applied to slices obtained from the trigeminal ganglia and superior cervical ganglia to calculate the percentages of β-gal expressing cells obtained when the Ncx-promoter is driving the Cre-cassette and is used to mediate excision leading to active β-galactosidase

transcription. Another calibration exercise was not done using these tissues because they were made up of cells which were more heterogeneous in both size and distribution compared with the DRG, therefore the errors associated with this counting method would have been improved (Hedreen, 1998)

Assessment of Cre activity (β-galactosidase-expression) in brain, spinal cord and enteric wall tissues was purely qualitative.

4.5 Results

4.5.1 Genotyping of Rosa-26R X Ncx-Cre offspring

Breeding of the homozygous Rosa-26 reporter strain (Rosa-26R) to the homozygous Ncx-Cre expressing strain did not lead to any overt developmental problems and all offspring survived well into adulthood. All offspring generated had the expected double-heterozygous genotype. This was demonstrated by using PCR primers to amplify both the Cre sequence (Figure 4.4) and to detect the Rosa-26R cassette (Figure 4.5).

Figure 4.4 demonstrates the efficacy if the Cre primer pair in discerning the Cre cassette (lanel and 2) when placed on either the Rosa-26R/Ncx-Cre background (lane 1) and on a Bl-6 background (the parent Ncx-Cre strain). Lane three shows no Cre band present in the original Rosa-26R parent strain.

The Rosa-26R stain could be genotyped to determine the number of mutant alleles (containing the β-gal neo reporter cassette) with a PCR protocol. Figure 4.5 illustrated the outcome when applied to four cases; a) Heterozygote animals with one copy of the wild type allele (550bp band) and one copy of the mutant reporter allele (250bp band), b) Homozygous Mutant animals with only the 250bp band, c) Wild-type animals displaying only the 550bp band (Figure 4.5)

4.5.2 Expression of Ncx-Cre in brain and spinal cord

Six animals were processed to determine the expression of Cre-recombinase in the brain and spinal cord. There was no expression in any brain structures or spinal cord including motor neurones of these animals (Figure 4.6). There was also no Cre-expression in any tissues taken from negative control animals (Rosa-26R) which were not crossed to the Cre-expressing strain (data not shown). This control was done to indicate whether the system was leaky i.e. there was some residual Cre-expression from self-excision events.

4.5.3 Assessment of the counting method

DRG obtained from the lumbar region (L1/2) were serially sectioned at 10μm and subjected to both a 3-D reconstructive count (von Bartheld, 2002; Williams and Rakic, 1998) as well as three independent estimated counts (methods 4.4.7) to determine a correction factor and estimation bias of the proposed counting method.

	3-d count	Estimate 1	Estimate 2	Estimate 3	Estimate Avg (1-3)	% Error
All neurones	5364	1592	1618	1615	1609	
All Cre	412	125	125	121	124	
% Cre	**7.68**	**7.84**	**7.73**	**7.49**	**7.69**	**0.07**
N52	1262	369	386	381	379	
N52/Cre	102	29	31	33	31	
% N52/Cre	**8.08**	**7.86**	**8.03**	**8.66**	**8.18**	**1.26**
Peri	4102	1226	1232	1234	1231	
Peri/Cre	310	96	95	88	93	
% Peri/Cre	**7.56**	**7.83**	**7.71**	**7.13**	**7.56**	**0.00**

Table 4. 1 Analysis of sampling method used

As can be seen from the data in table 4.1 above, there was a slight overestimation in the number and percentages of DRG neurones using the method outlined. The error generated raged between 0% and 1.3% of the true figure and was normally distributed with respect to the actual figure (determined by the 3-dimentional reconstructive count).

4.5.4 Expression of Ncx-Cre in Dorsal root ganglia

Between three and six DRG drawn from a minimum of three animals in each case were assessed for expression at the cervical, upper thoracic (TH3/4), lower thoracic (TH7/8), lumbar (L4/5), and sacral (S1/2)levels (Figure 4.7).

No expression was found at the cervical level (n = 3) as well as in the upper thoracic DRG (TH3/4) (n =3) (Figure 4.7).

There was limited expression present in the lower thoracic region (TH7/8) which demonstrated average levels of 13.7 ± 0.6% (n = 4) of all neurones (Figure 4.10). Similar

expression levels were also noted in the lumbar region (L4/5). Here the number of positive neurones amounted to 11.1 ± 0.5% (n = 4) of the total (Figure 4.8). At the sacral level, expression was again present but the level was reduced in comparison to the lumbar and lower thoracic levels, only achieving 5.7 ± 0.8% of all neurones counted (n = 4) (Figure 4.9).

The neurones expressing Cre were also further subdivided into populations co-expressing neurofilament (a marker for large diameter neurones) and Peripherin (a marker for small-diameter neurones). This was done immunohistochemically as outlined above and the Cre-expressing neurones in a section were compared with the next section in the series to determine co-expression of Cre and either peripherin or neurofilament. This allowed the subdivision of the Cre-expressing neurones shown in table 4.2. No population of cells was found to be unlabelled by the histochemical protocol used as demonstrated by a comparison of the total neurones counts using the sequence of slides stained for neurofilament/peripherin compared to the slides stained for β-galactosidase and neutral red (data not shown).

In the counting of the slides labelled for both neurofilament and peripherin, any cells which were double-labelled were allocated according to their size to either the small diameter group (under 25μm in cross-sectional diameter) or the large diameter group (over 25μm).

The data in table 4.2 demonstrates that the maximal level of Cre-expression achieved in any DRG using the Ncx promoter sequence to drive the cassette occurred at the lower thoracic level. Here 13.7 ± 0.7% of all neurones expressed the functional Cre-recombinase protein. Of this population, 15.1 ± 1.6% of all peripherin positive nociceptors were found to express Cre whilst 8.5 ± 0.8% of larger diameter neurones made up the remaining Cre-expressing cells.

DRG	N52	N52/Cre	Peri	Peri/Cre	Total
Th7/8 n=4	22.5 ± 1.0	2.1 ± 0.6	65.2 ± 4.1	11.6 ± 0.7	101.4 ± 1.6
L4/5 n=4	20.9 ± 0.8	3.3 ± 0.4	67.6 ± 1.0	7.8 ± 0.6	99.6 ± 0.6
S1 n=4	19.1 ± 1.0	1.6 ± 0.6	70.3 ± 3.9	4.1 ± 0.9	96.1 ± 2.6

Table 4.2 Quantified Ncx-Cre expression levels in DRG

Table 4.2: Percentages obtained for neuron subtypes for Ncx-Cre expressing mouse DRG (TH7/8; Thoracic level 7/8, L4/5; Lumbar level 4/5, S1; sacral level 1, N52; marker for large diameter neurones, Peri; maker for small diameter nociceptors, Cre; neurones expressing functional Cre-protein under the control of the Ncx-promoter).

DRG isolated from the Lumbar region, specifically L4/5 which are mainly composed of neuronal bodies whose terminals travel via the Sciatic nerve to terminate in the hind limbs show slightly less Cre expression (11.1 ± 0.5%). The sacral DRG that innervate the posterior area and upper tail region also express Cre but at even lower levels (5.7 ± 0.7%).

4.5.5 Expression of Ncx-Cre in the Sympathetic ganglia in the Ileum

The ileum which makes up the lower part of the small intestine proximal to the colon is well innervated with sympathetic neurones and ganglia (Figure 4.3). These tissues were investigated for the presence of Cre. As shown in figures 4.11 and 4.12, the neurones innervating these tissues expressed the functional Cre-protein in abundance when driven by the Ncx-promoter. Positive expression was observed in neurones in both the myenteric (Figure 4.11a) and the sub-myenteric (Figure 4.12) plexii. However there was no detection in neurones or neuronal projections within the mucosal layer (n=4) (Figure 4.13). The Myenteric plexus (Figure 4.9b) as well as the sub-myenteric plexus (Figure 4.13) were also immunolabelled for Peripherin and were found to be positive for peripherin expression. The distribution of peripherin-positive neurones closely followed that of the Cre expression pattern in the myenteric network (Figure 4.11a) as well as the sub-myenteric network (Figure 4.13).

4.5.6 Expression levels in Trigeminal and Superior cervical ganglia

These tissues which form part of the sympathetic nerve system were also found to contain Ncx-Cre protein (as assessed by the presence of active β-galactosidase in the reporter cross) in the adult mouse (Figures 4.14 & 4.15).

Quantification of these tissues showed that only 1.4 ± 0.1% of trigeminal neurones expressed Cre under the Ncx-promoter (n = 4 ganglia) (Figure 4.14). The superior cervical ganglia on the other hand showed good expression with over half of the neurones expressing Cre, 51.4 ± 2.6% (n = 4). (Figure 4.15)

Figure 4.4 Ncx-Cre PCR analysis

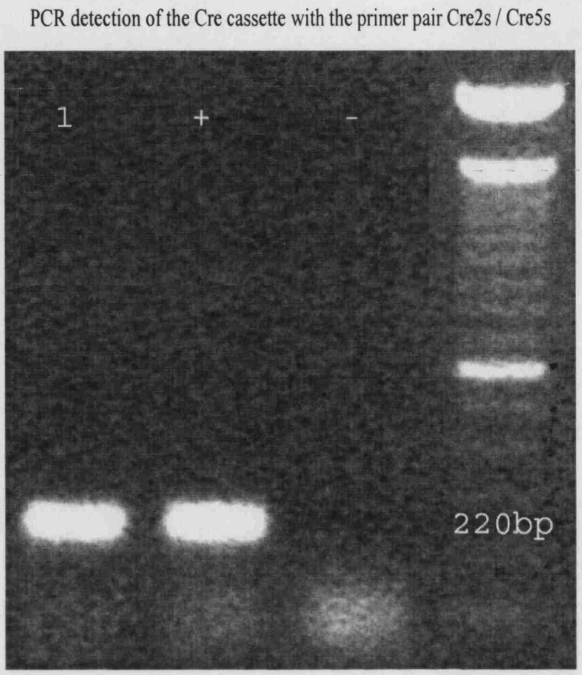


Figure 4.4: Agarose gel showing the PCR products obtained from the application of Cre-specific primers to genomic DNA isolated from; Lane 1) Rosa-26R/Ncx-Cre double heterozygote mouse, Lane 2) Ncx-Cre homozygote mouse, Lane 3) Rosa-26R homozygote mouse. The presence of Cre sequence is indicated by the presence of a 220bp PCR product. Both the Ncx-Cre parental strain and the Ncx-Cre/Rosa-26R cross exhibit this band. The Rosa-26R parental strain does not give a positive result demonstrating the specificity of the primer pair used.

Figure 4.5 ROSA-26R PCR analysis

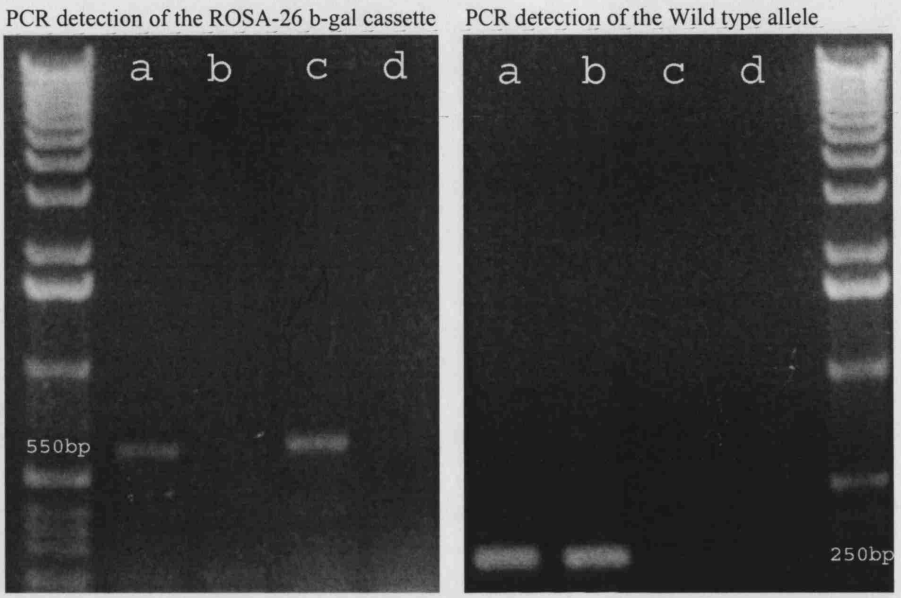


Figure 4.5: Agarose gel showing the PCR products obtained from the application of Rosa-26R-specific primers to genomic DNA isolated from; Lane a) Rosa-26R heterozygous mouse (250 bp & 550bp bands), Lane b) Rosa-26R homozygous mouse, Lane (only 250bp band) c) Wild-type mouse (only 550 bp band), Lane d) negative control (no band). The priming location of the primers used is shown in Figure 4.2. In the floxed gene the primer pair 883/315 gives a band of 250bp, the presence of the inserted cassette disrupts the product from the primer pair 883/316 (550bp), which is now too far apart to give a product under the reaction conditions used. In the wild type gene however, the 315 primer cannot bind as it is complementary to sequences contained in the β-galactosidase cassette. The PCR primer pair 338 and 316 is still close by and gives a band of 550bp in the wild type allele.

Figure 4.6 β-Gal staining in adult brain of Ncx-Cre X Rosa-26R cross

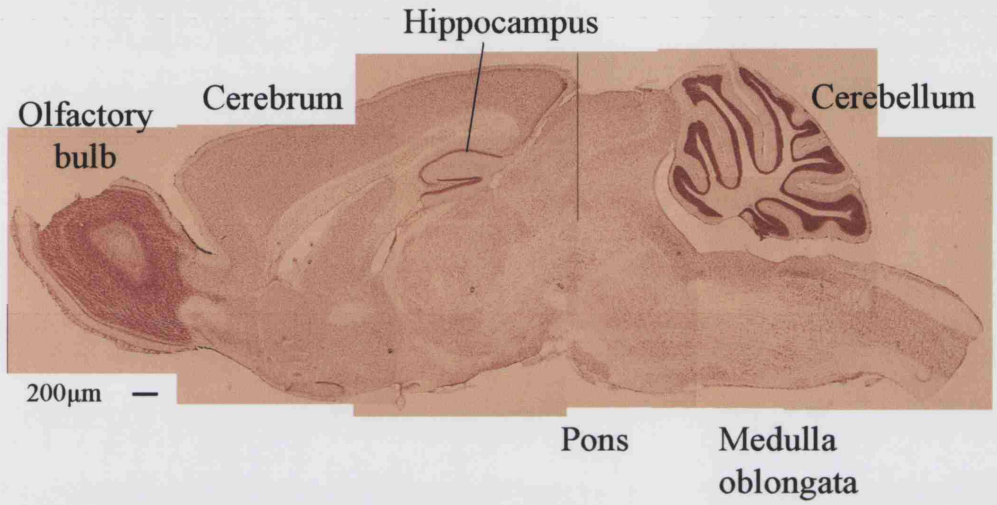


Figure 4.6: No detection of functional β-Galactosidase was observed in any brain structure of adult animals (n=5). This shows that there is no active Cre-recombinase in these tissues in the Ncx-Cre X Rosa-26R strain.

Figure 4.7 β-Galactosidase staining in adult Ncx-Cre X Rosa-26R DRG

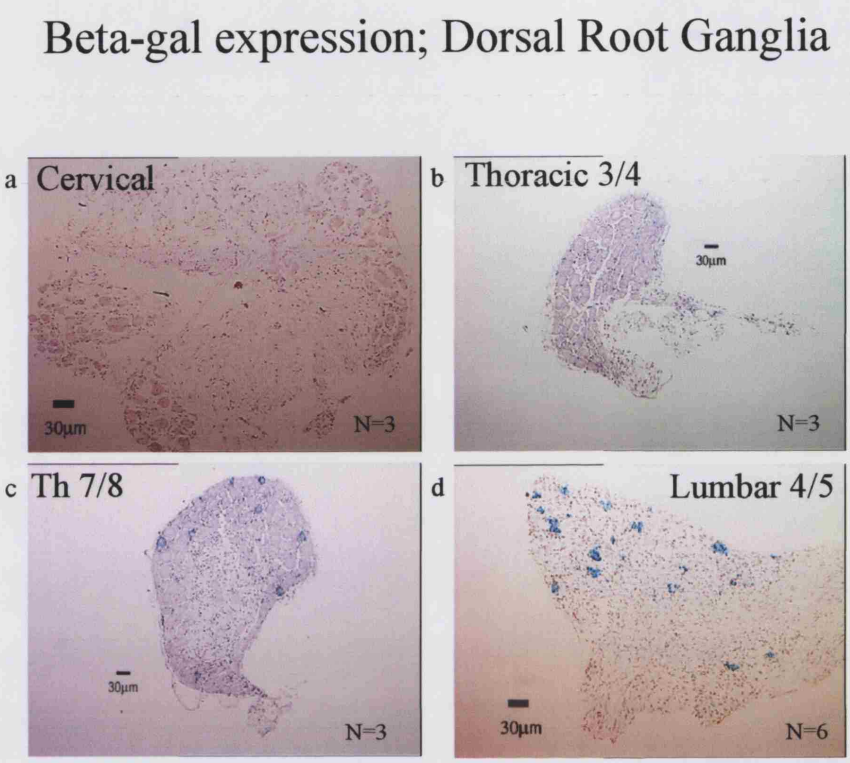
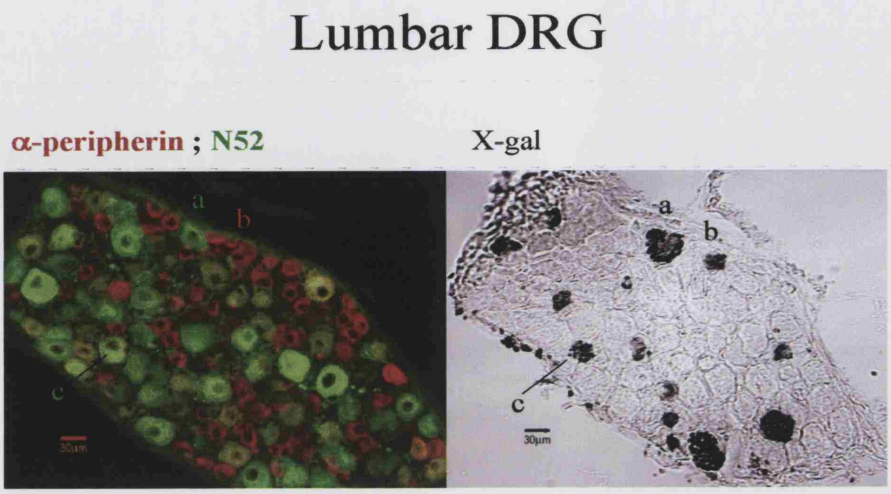


Figure 4.7: Functional Cre-recombinase presence in DRG from various spinal levels exemplified by active β-galactosidase presence in cells (Blue); a) Cervical DRG section showing no functional β-gal expression, b) no expression in the upper thoracic DRG, c) limited expression in both Thoracic 7/8 and d) Lumbar 4/5 DRG.

Figure 4.8 β-galactosidase expression in L4/5 DRG of Ncx-Cre X Rosa-26R mice, comparison slide stained for peripherin and N52



β-galactosidase expression by N52 +ve cells (a & c) or peripherin +ve nociceptors (b)

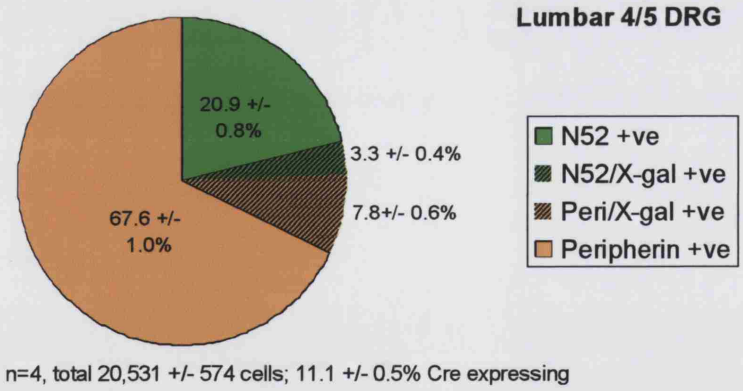
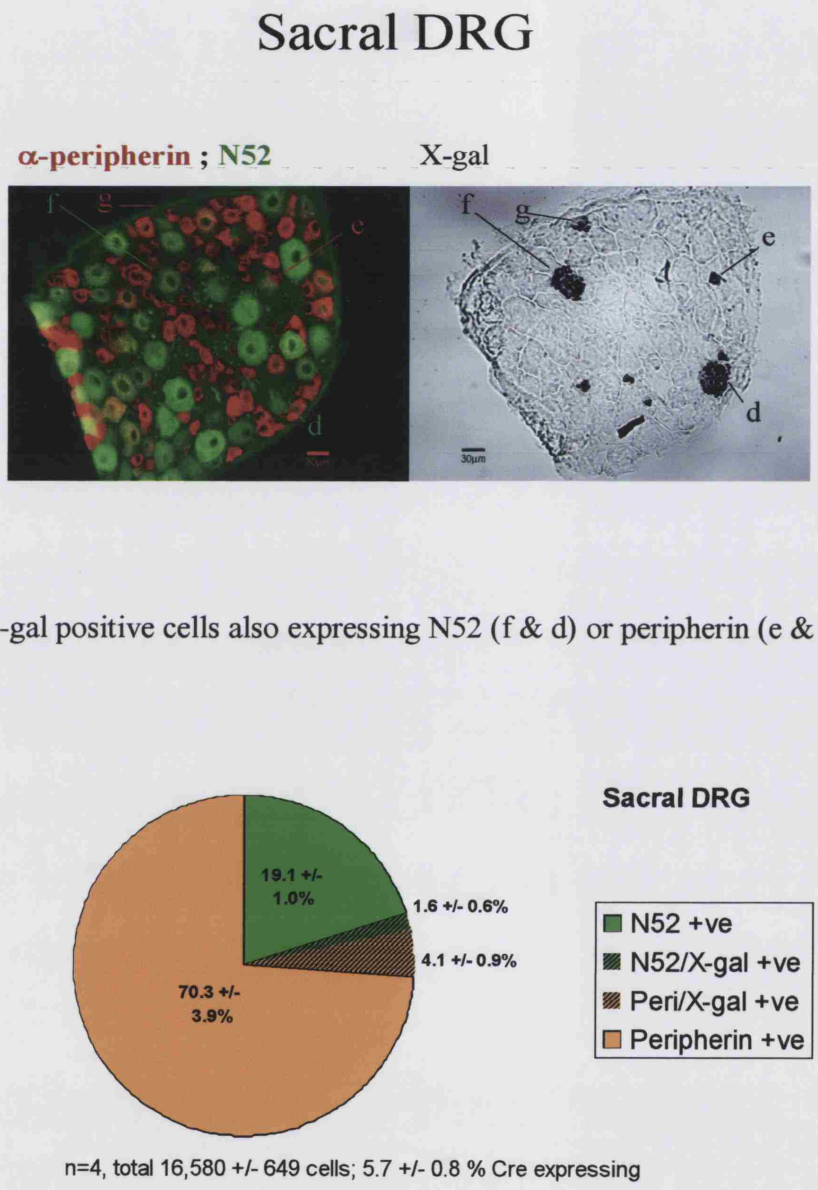


Figure 4.8: a) Comparison between two adjacent 10µm section of Lumbar DRG to allow the split of the β-gal positive neurones (right picture) here shown in black into neurofilament positive (green cells) and peripherin positive (red) subpopulations, b) chart showing expression in the two cell populations of lumbar DRG

Figure 4.9 β-galactosidase expression in S1/2 DRG of Ncx-Cre X Rosa-26R mice, comparison slide stained for peripherin and N52

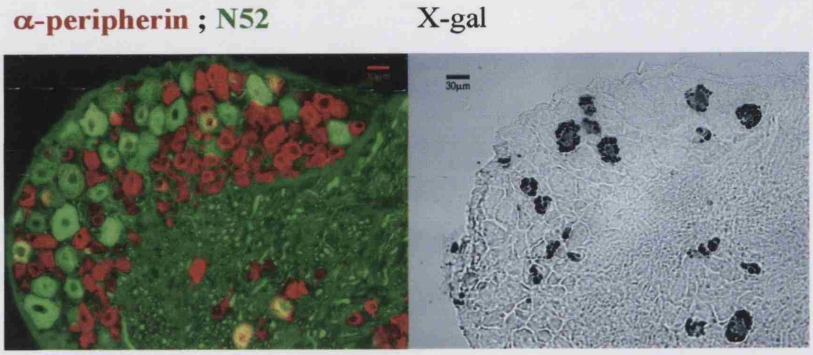


β-gal positive cells also expressing N52 (f & d) or peripherin (e & g)

Figure 4.9: a) Serial sections of sacral DRG showing expression pattern of B-galactosidase.b) Chart showing large diameter neurones (Green) of which a percentage express β-gal (shaded green) and peripherin positive small-diameter neurones (orange) of which the shaded fraction represents the β-gal expressing subpopulation.

Figure 4.10 β-galactosidase expression in Th7/8 DRG of Ncx-Cre X Rosa-26R mice, comparison slide stained for peripherin and N52

Thoracic DRG



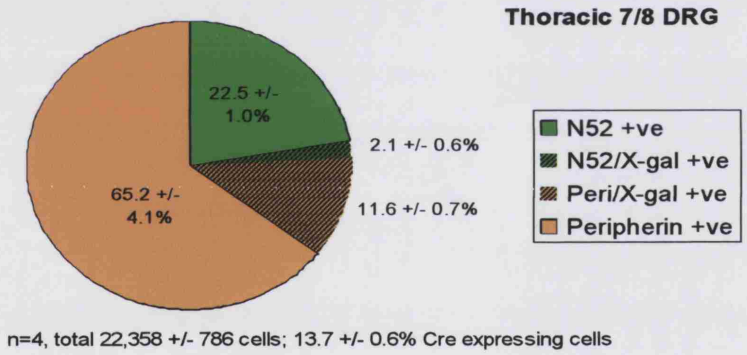


Figure 4.10: a) The β-galactosidase expression pattern in adult mouse DRG at the thoracic level 7/8 taken from Ncx-Cre X Rosa-26R mice. b) Chart showing the subdivision of all DRG neurones at this level into large and small diameter neurones and their respective β-galactosidase expressing subpopulations. The presence of functional β-galactosidase indicates that the lox-P sites of the β-galacotosidase gene in these cells have undergone Cre-mediated excision.

Figure 4. 11 β-galacatosidase expression in sympathetic ganglia in the gut serosa of adult Ncx-Cre X Rosa-26R mice

Myenteric plexus;

longitudinal section (n=3)

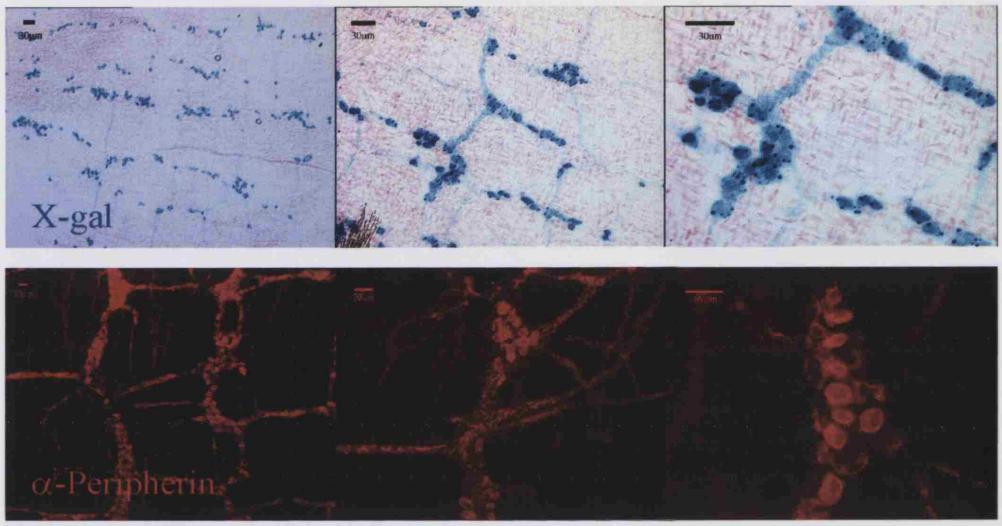


Figure 4.11: a) β-galactosidase expression in neurones of the myenteric ganglia of the adult Ncx-Cre x Rosa-26R mouse. b) Peripherin epitope expression by the majority of myenteric neurones. The majority of the neurones in the myenteric ganglia of this cross have undergone Cre-mediated excision leading to the presence of functional β-galactosidase being present.

Figure 4.12 β-galactosidase expression in sympathetic ganglia in the gut lumen of adult Ncx-Cre X Rosa-26R mice

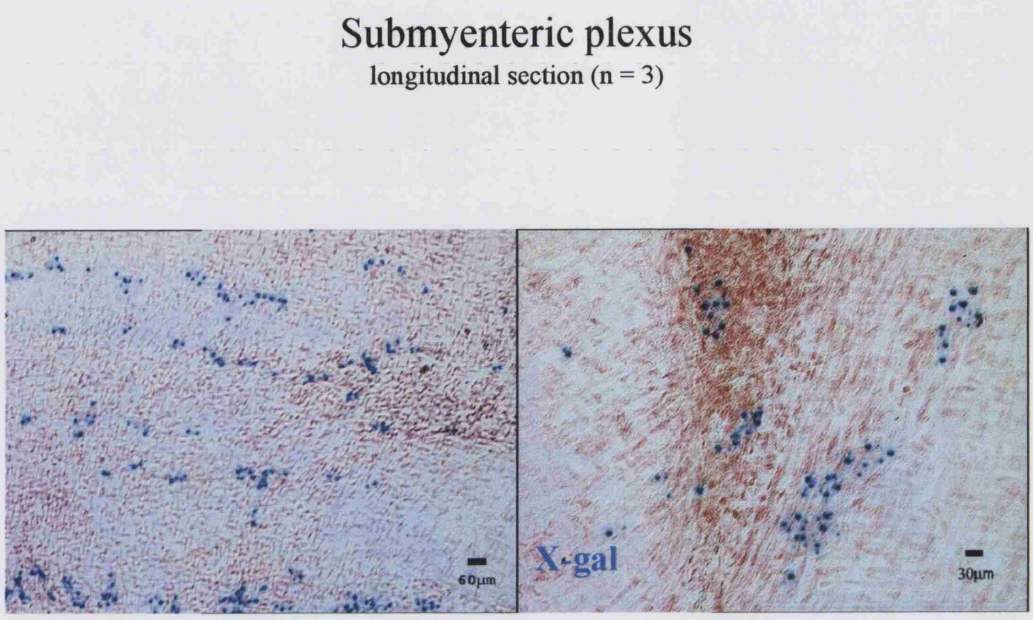


Figure 4.12: a layer of muscle taken from the ileum wall showing the abundant expression of β-galactosidase in the sub-myenteric plexii of the adult Ncx-Cre x Rosa-26R mouse. This demonstrates that Cre-mediated excision events have occurred in these cells under the control of the Ncx promoter.

Figure 4.13 β-galactosidase expression; ileum cross-section

Myenteric/submucosal plexi;
Transverse section (n = 3)

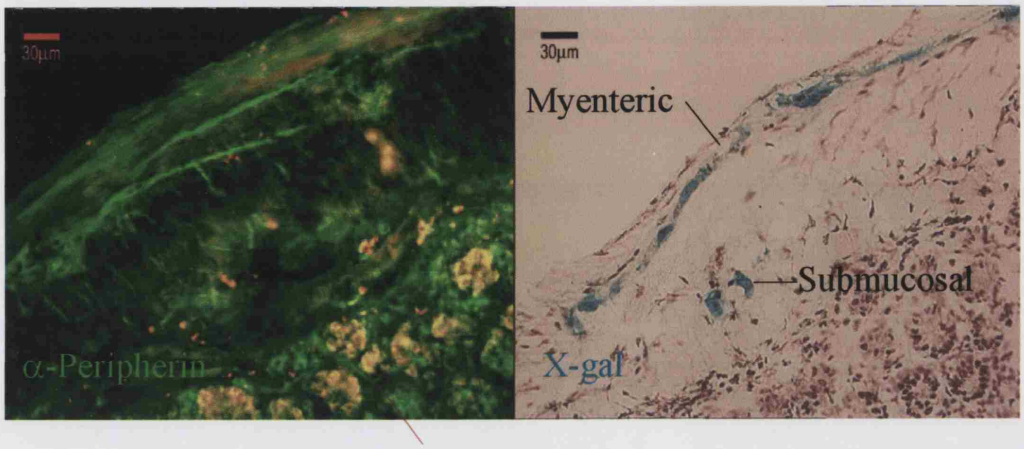


Figure 4.13: 10µm serial sections showing the cross-section of the ileum wall. a) Peripherin expression (green). b) functional β-galactosidase expression in both the Myenteric and Sub-myenteric (sub-mucosal) ganglia and nerves but not in the mucosal layer can be seen.

Figure 4.14 β-galactosidase expression; sympathetic ganglia (Trigeminal) of adult Ncx-Cre x Rosa-26R mice

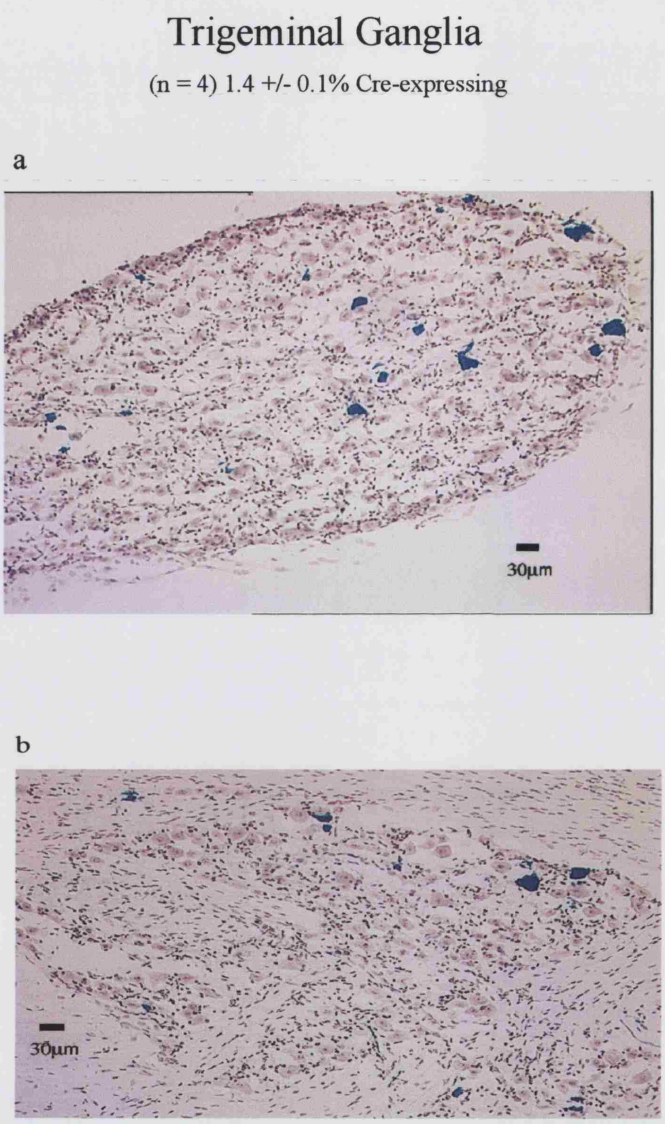


Figure 4.14: 10µm sections of trigeminal ganglia harvested from Ncx-Cre x Rosa-26R mice showing the β-gal expression pattern achieved. An average of 1.4 ± 0.1% of trigeminal neurones expressed the functional enzyme indicating the presence of fuctional Cre-recombinase. a) Longitudinal view b) transverse view.

Figure 4.15 β-Galactosidase expression sympathetic ganglia (SCG) of adult Ncx-Cre x Rosa-26R mice.

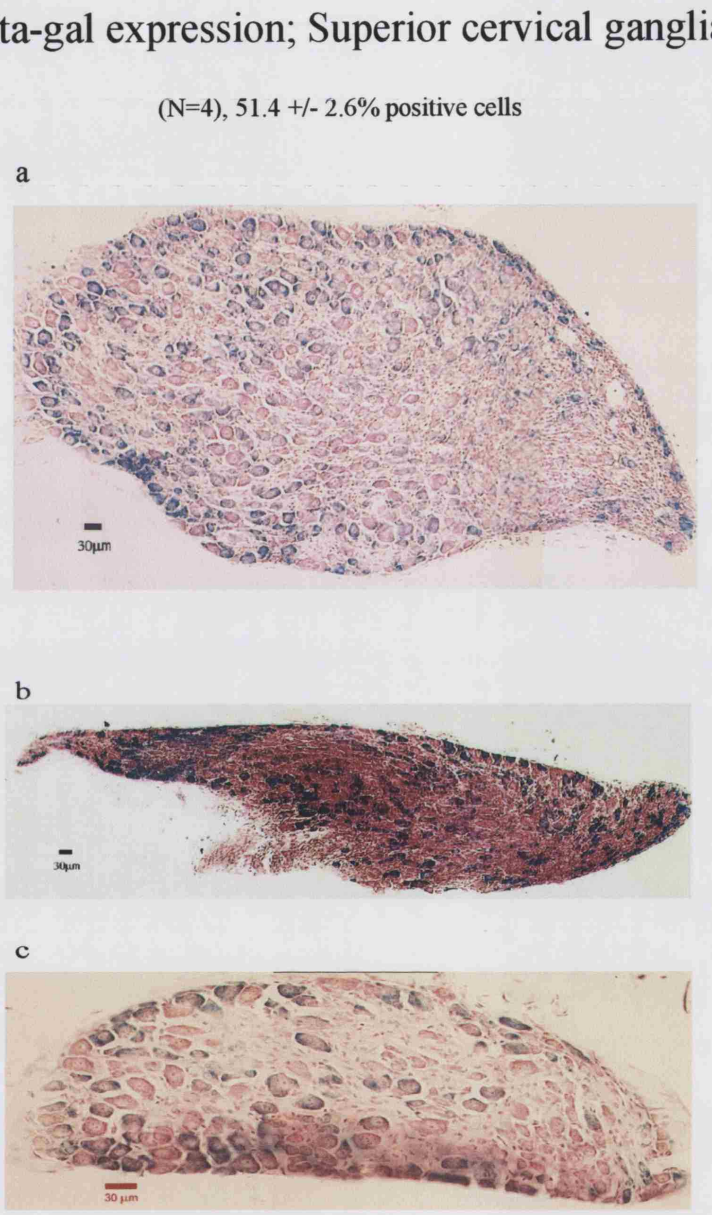


Figure 4.15: Expression pattern of β-galactosidase in adult Ncx-Cre x Rosa-26R mice superior cervical ganglia a) parasagittal section b) sagittal section c) transverse section. Approximately half of all the neurones are positive for β-galactosidase indicating a successful Cre-recombinase mediated excision event has occurred.

4.6 Discussion

The regulated expression of genes is an important method by which the interactions of a complex system can be teased apart. The development of transgenic animals which have particular sequences either upregulated or ablated have increased our knowledge of pain pathways. Many genes have been implicated in nociception. However, the need to engineer transgenic systems which allow the control of putative nociceptive genes both temporally and spatially in sensory neurones remains in its early stages due to the dearth of nociceptive specific promoters which can be used to drive efficient expression of genes of interest.

The investigation of the Ncx/Hox11L.1 gene was done to determine the expression pattern and to quantify the level of expression attained using its promoter sequence in sensory neurones and specifically nociceptive neurones in the dorsal root ganglia (DRG).

The DRG is the major organizational structure for the somata of sensory neurones innervating the periphery. These neurones are responsible for the detection of sensory input and information relay up to the dorsal horn for further processing. As the first link in the pain pathway, it is an important site for the action of analgesics. Specific targeting of these neurones will play an important role in the development of more effective intervention technologies to alleviate pain, in both its acute and chronic form.

The expression pattern of Cre-recombinase under the control of the Ncx promoter region was assessed using a β-galactosidase expressing reporter strain ROSA-26R. This reporter strain has been shown to ubiquitously express β-galactosidase from embryonic stage E9 (Soriano, 1999) when Cre is driven by the ROSA26 promoter itself. The Ncx gene itself has been shown to be active from E9.5 and maximal at E12.5 (Hatano et al., 1997b). This means that the β-galactosidase gene is available for expression as soon as the Cre enzyme promoter drives the excision event.

The level of expression in the DRG was not equivalent at all levels investigated. In fact, the expression of the Cre gene could not be detected in the cranial, caudal or upper thoracic DRG. Only DRG located after thoracic level 7/8 and before the caudal level showed any expression of Cre. Furthermore the average expression level amounted to only 10.1 ± 0.6% of all neurones present in the DRG. There was also significant variation

in the expression levels ranging from a high of 13.7 ± 0.6% in lower thoracic DRG to a low of 5.7 ± 0.8% in the sacral DRG.

When this population of neurones expressing the Cre-recombinase was further investigated, it was determined that of the Cre-expressing neurones, an average of 75.6 ± 4.5% were small diameter, peripherin-positive sensory neurones (the nociceptor subpopulation) and the remaining 24.4 ± 4.5% were large-diameter neurones which are mainly mechanosensitive and propriosensitive in nature. The exact proportions again depended on what level of DRG was quantified, with the lower thoracic DRG displaying the highest contribution of Cre-expressing small diameter nociceptors (84.7 ± 0.6 %) as opposed to either the lumbar or sacral regions (70.2 ± 0.5 % and 71.9 ± 0.8 % respectively).

The expression pattern in the gut walls showed that nearly all of the neurones belonging to adult myenteric and sub-myenteric ganglia express Cre-recombinase under the expression of the Ncx promoter as exemplified by the presence of functional β-galactosidase. This supports earlier evidence from embryonic studies showing that Ncx could be detected strongly in the intestine walls of embryos (stage E13.5) (Hatano et al., 1997b).

This group further suggested that the expression pattern, as determined through the use of *in situ* hybridisation techniques in mice embryos, included the adrenal glands, DRG, cranial ganglia, and sympathetic ganglia although in cranial ganglia they disappeared by E17. Ncx expression in adults was located to the intestine and the adrenal glands by RT-PCR (Hatano et al., 1997b).

Analysis of the trigeminal ganglia in adult mice expressing Cre-recombinase under the control of the Ncx promoter confirmed that the level of expression of Cre-recombinase in these adult cranial ganglia is almost absent with under 2% expressing active β-galactosidase. This again supports the RT-PCR data provided by Hatano et al (1997b). This can be understood as the vast majority of sensory neurones which form the trigeminal ganglia are placode-derived with only a small proportion being neural crest derived. The cells derived from the neural crest become either glial cells or sensory neurones in the terminally differentiated trigeminal ganglia (Baker et al., 2002). The result of this work indicated that less that 2% of the neurones in the adult trigeminal ganglia are

derived from neural crest progenitor cell assuming that they all continue to express Ncx into adulthood.

The expression in adult sympathetic ganglia was assessed by extracting and quantifying the level of β-galactosidase expression in the superior cervical ganglia. This sympathetic ganglion receives input mainly from the upper trunk region and is thought to be a conglomeration of at least four cervical ganglia. It is located near to the carotid sheath and forms part of the cervical sympathetic ganglionic chain together with the inferior and median cervical ganglia. This ganglion showed good expression of the β-galactosidase with over half the neurones being positive. This result indicated that a substantial proportion of neurones contained within the superior sympathetic ganglia are differentiated from the neural crest.

Hatano et al suggested that because they could not detect the presence of mRNA for Ncx in migrating neural crest cells, it therefore primarily plays a role in the proliferation and the differentiation stages. This was supported by their finding that the maximal levels of Ncx were to be found at E12.5 when these processes are occurring in the embryo, and that Ncx expression in the adult is limited to the intestine and the adrenal glands where it is probably continuing to provide support into adulthood (Hatano et al., 1997b).

The development of the megacolon in Ncx knock-out mice indicates strongly that Ncx is an important protein in the development of the mature innervation pattern of the enteric nervous system (Hatano et al., 1997a). Additionally, it was reported that the precise morphology of the abnormality differed from the megacolon induced by the deletion of other genes such as c-Ret (Schuchardt et al., 1994) or its ligand GDNF (glial cell derived neurotrophic factor) (Sanchez et al., 1996; Pichel et al., 1996) or the endothelin-B receptor (Hosoda et al., 1994) and its ligand endothelin-3 (Baynash et al., 1994), or the dominant megacolon gene *dom* (Lane and Liu, 1984; Kapur et al., 1996).

At present there is little evidence available to indicate the mechanism of the Ncx protein in the establishment of a mature enteric innervation pattern. However the absence of Ncx did not prevent the migration of neural crest cells as seen in the c-ret knockout animals or the Endothelin-B receptor knock-out animals (Kapur et al., 1995).

Both of these genes have been implicated in the human congenital megacolon associated with Hirshsprung's disease which is characterised by an absence of enteric

neurones in the colon (Edery et al., 1994; Puffenberger et al., 1994; Romeo et al., 1994) and an increase in nerve fibres from extrinsic sources(Facer et al., 2001). The remaining nerves innervating the aganglionic region of the gut associated with this disease have been shown to express strong TRPV-1 immunoreactivity which has been well correlated to a subclass of heat and chemically activated sensory neurones (Ohtori et al., 2003). The possibility that the Ncx-Expressing subpopulation of DRG neurones could be the originator of these hypertrophic nerve fibres remains to be investigated.

Added together these results demonstrate that only a small proportion of neurones in the DRG express Ncx, therefore the Ncx promoter is not a suitable choice to drive gene ablation with Cre-recombinase in all nociceptors.

Chapter 5: Nociceptor-specific gene deletion with an $Na_V1.8$–Cre expressing strain; The affect of BDNF gene deletion in $Na_V1.8$ expressing cells on physiological and inflammatory pain states.

5.1 Synopsis

The use of current HSV-based viral transduction methods to achieve genetic modulation of sensory neurones did not achieve the high level of transduction (chapter 2) required in any particular subset of neurones precluding their use in functional studies. The use of antisense technology, whilst of a high efficiency, is also limited due to the inability of the protocols employed (exemplified in chapter 3) to specifically target subsets of sensory neurones. Alternative strategies are therefore required to study gene expression specifically in nociceptors.

The use of defined Cre-expressing transgenic lines would allow a controlled ablation event of floxed genes in cells expressing Cre-recombinase. Of the two transgenic lines available, the Ncx-Cre line did not display a high level of expression specifically in small diameter sensory neurones (chapter 4). A second transgenic strain, the $Na_V1.8$–Cre line (Stirling et al., 2003), was therefore trialled.

The development of this nociceptive-specific Cre strain ($Na_V1.8$–Cre) has opened the possibility of studying the function of broadly expressed genes in pain pathways. The limited expression of the Cre-recombinase avoids detrimental developmental abnormalities encountered with unconditional knock-outs

The $Na_V1.8$ –Cre line was crossed with a Brain derived neurotrophic factor (BDNF) floxed line to characterise the involvement of this neurotrophin in various pain pathways. BDNF is an important neurotrophin during development and unconditional knock-out of this gene leads to lethality.

BDNF is known to play an important role in pain pathways. Previous studies with BDNF sequestering molecules have demonstrated that its removal can have profound effects on various measures of peripherally and centrally mediated pain states. The source of this BDNF is still unclear however due to the imprecise action of sequestering molecules. This chapter presents evidence for the impact of BDNF derived from a subset

of DRG sensory neurones, the $Na_v1.8$-positive neurones, in the determination of baseline levels of pain and the development of inflammatory pain *in vivo*.

The hypothesised role of BDNF in the trasmission of nociceptive signals in the dorsal root ganglia requires its production and release in a coordinated manner. A possible source of this BDNF is the central (dorsal horn) projections of neurones that respond nociceptive stimuli at the periphery (small diameter nociceptors). In order to tease apart this pathway it is necessary to specifically remove the production of BDNF in defined subsets of neurones (nociceptors) to determine whether they form the source of the BDNF.

The new cross, in which the BDNF gene was removed specifically in $Na_V1.8$ expressing neurones (coinciding with the majority of nociceptors), was then assessed to determine the drop in BDNF expression in the DRG and the consequent alterations in behaviour during both infamed and non-inflamed conditions. This data should help to validate the role of nociceptor-derived BDNF *in vivo*.

5.2 Introduction

Brain-derived neurotrophic factor (BDNF, a member of the family of neurotrophins) is essential for the survival and maintenance of peripheral sensory neurones (Ernfors et al., 1994; Jones et al., 1994). The neurotrophin family also includes the members NGF, NT-3 and NT4/5 which act in concert to support the development of subpopulations of DRG neurones and continue to do so into adulthood.

Studies have shown the importance of BDNF in pain pathways, specifically BDNF expression levels in the nervous system are altered in a number of pain models including peripheral inflammation (Cho et al., 1997a; Cho et al., 1997b), axotomy and nerve injury/ neuropathic pain paradigms (Zhou et al., 2000; Zhang et al., 2000; Ha et al., 2001; Cho et al., 1998).

5.2.1 BDNF gene transcripts

Studies on the rat BDNF gene have demonstrated that there are four differentially regulated promoters. Each promoter directs the production of an mRNA species containing a unique 5' exon (1-4) and a common 3' exon (5) that encodes the mature protein (Michael et al., 1999). This paper also showed that the different 5' exons confer tissue specificity on the various mRNA transcripts.

Other studies have shown that the expression and ratio of different transcript mRNAs is dynamic (Kokaia et al., 1994) and different states can induce the production of one or other mRNAs through the use of these specific promoters (Kim et al., 2001).These alternate promoters may provide a mechanism by which BDNF can be used by the nervous system to alter the nociceptive processing within the nervous system in response to inflammation or nerve injury. A possible mechanism, dependant on the differing binding affinities of the BDNF subtypes to the truncated or full length TrkB receptor, may be in operation with one or the other being transcribed preferentially during inflammation or nerve injury (Tonra, 1999).

The BDNF gene is thought to be an immediate early gene, with the exon 4 version in particular reaching maximal levels at one hour post noxious stimulus (Lauterborn et al., 1996). This suggests that the BDNF protein may be exerting its effects during the initial

phases of chronic noxious stimulation such as inflammation. However, the finding that BDNF is packaged in rough vesicles (Michael et al., 1997) argues against this as these vesicles appear to bind during the later stages of the inflammatory response, releasing their contents into the dorsal horn after the smooth vesicles release glutamate. The exact nature of the various BDNF variants action during inflammation and their timing remains unclear.

5.2.2 Trk receptors and BDNF

The Trk family (TrkA, TrkB and TrkC) consists of high-affinity receptors for each binding preferentially to one or other neurotrophin. BDNF preferentially binds to TrkB (which also binds weakly to NT-4/5), but of the two recorded isoforms of TrkB (Klein et al., 1990; Middlemas et al., 1991), only the full length TrkB (FL.TrkB) isoform has the associated protein tyrosine kinase activity (PTK) (Klein et al., 1990). This PTK property when activated by the binding of BDNF is responsible for triggering a kinase cascade leading to the phosphorylation and activation of transcription factors affecting gene transcription.

In contrast to the FL.TrkB molecule, the truncated isoform (TR.TrkB) lacking the PTK domain, has been proposed to act as an inhibitory modulator of BDNF-responsiveness mediated by FL.TrkB. It appears that TR.TrkB activation may preferentially bias BDNF signal transduction in its favour over the alternative pathway mediated by the full length isoform (Eide et al., 1996). TR.TrkB is predominantly located in the spinal dorsal horn (Lee et al., 1999) where it may act to depress BDNF-modulated activity.

In their paper Yajima *et al* 2001 reported that the thermal hyperalgesia induced by nerve ligation could be abolished by removing the BDNF in the dorsal horn but not by removing NT-4 demonstrating a role for BDNF in thermal hyperalgesia. Further, these BDNF mediated effects within the dorsal horn were protein-kinase directed, as shown by the application of K-252a, a protein kinase inhibitor which reversed the effect.

However it remains that these effects depend of the interaction with the full-length isoforms, whereas it has been shown that the truncated form is also found in the dorsal

horn (Yacoubian and Lo, 2000; Baxter et al., 1997) which suggests that another transcription-independent mechanism may also be in operation.

5.2.3 BDNF expression *in vivo*

Unlike other neurotrophins BDNF is constitutively present in sensory neurones and expressed by a subpopulation of small-diameter sensory neurones (Figure 5.1) with unmyelinated axons terminating in the superficial laminae of the dorsal horn (Ernfors et al., 1990).

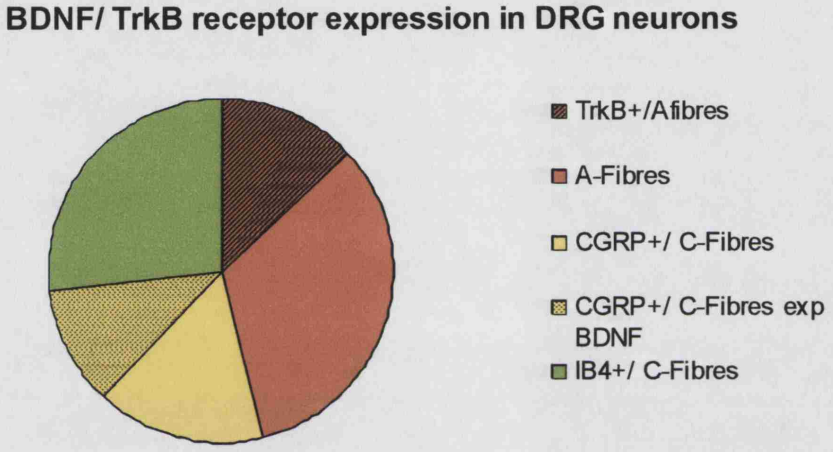


Figure 5. 1 BDNF/TrkB expression pattern under physiological conditions

(Thompson et al., 1999)

BDNF undergoes anterograde transport to the dorsal horn where it is associated with synaptic vesicles of nociceptive neurones and presumably is released onto first-order spinal neurones (Michael et al., 1997). The expression of BDNF in the developing and mature neural population in the DRG suggests that BDNF may play an autocrine function as well. This has been shown *in vitro* (Acheson et al., 1995).

Evidence of BDNF acting as a possible neurotransmitter or neuromodulator within the dorsal horn of the spinal cord however, is increasing (Thompson et al., 1999; Kerr et al., 1999). BDNF can be transported anterogradely to the dorsal horn of the spinal cord (Tonra et al., 1998), where it may modulate the NMDA-evoked response (Cho et al., 1997a) and nociceptive sensory inputs in the spinal cord following inflammatory pain

(Kerr et al., 1999). The source of this BDNF has not been adequately demonstrated however, and this chapter presents evidence to answer this question.

The dorsal horn activity of BDNF has also been inferred using BDNF-sequestration protocols (Thompson et al., 1999). This group presented evidence to argue for the spinal modulation of BDNF in an activity dependant manner. The effects of BDNF in inflammatory pain states were shown to be reversible by the administration of a BDNF sequestration molecule. However these studies have relied on the intrathecal administration of trkB-IgG fusion proteins that sequester BDNF.

These sequestration protocols suffer from the disadvantage that these molecules are not able to discriminate between BDNF and the closely related neurotrophin-4/5 (NT-4/5) molecule. This chapter goes some way in trying to establish which of the observed effects, if any are attributable to the actions of BDNF specifically.

Other studies have been carried out to try and tease apart the differences using BDNF or NT-4 knock-out mice(Heppenstall and Lewin, 2001), but these studies have only been done in p4-p7 neonatal mice due to the inability of these mice to survive past the second post-natal week. The plasticity of the neonatal nervous system is well documented and as such the results obtained from these studies may only partially reflect the adult situation as deletion of one or other of the neurotrophins may switch BDNF-dependant subsets of adult neurones from BDNF to NT-4 or vice versa.

As many developing neurones have multiple neurotrophin requirements it is not known at present how DRG neuron populations are affected by a loss in BDNF during development. BDNF knock-out animals (-/-) die during the second postnatal week precluding the assessment of the role of BDNF in the mature sensory system *in vivo* (Rios et al., 2001).

This study provides some evidence regarding the neurotrophic requirements of DRG Neurones for BDNF derived from $Na_V1.8$-expressing cells (nociceptors) after E15 using a $Na_V1.8$-derived nociceptor-specific promoter to drive Cre expression (Stirling et al., 2003) in this population of cells.

The majority of DRG cells which express BDNF possess the TrkA receptor, which binds NGF with a high affinity. Furthermore, NGF has been shown to upregulate the expression of BDNF in these cells (Michael et al., 1997; Apfel et al., 1996; Cho et al., 1997b). This upregulation of BDNF expression (both in quantity and number of cells

expressing BDNF *de novo*) is also seen in inflammatory conditions where NGF-mediated effects are apparent (Figure 5.2). The majority of BDNF expressing cells also coexpress the neuropeptide substance P (subP) which is co-released with it in the dorsal horn in an activity dependant manner where they may function to modulate glutamate-mediated fast nociceptive transmission.

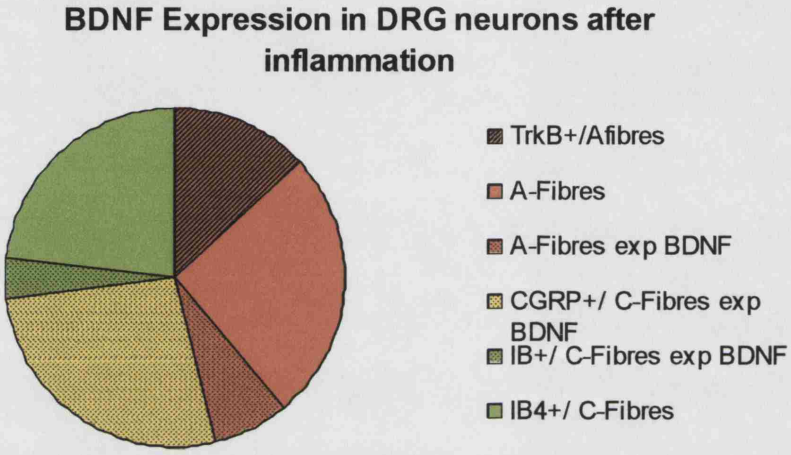


Figure 5. 2 BDNF/TrkB expression after inflammation

(Thompson et al., 1999)

BDNF mRNA has also been detected in the deeper dermal layers of the skin, where low-threshold mechanoreceptors predominate. This suggests that, unlike NGF, which is well expressed in the superficial dermal layers where nociceptors afferent terminals are located and appears to support these neuronal fibres, BDNF may not offer trophic support for these populations. In contrast to this, BDNF removal appears to allow the invasion of nerves from dendritic structures of the deeper dermal layers to more superficial targets (Rice et al., 1998). This evidence may support a role for BDNF in neuropathic pain paradigms where target-derived BDNF may be affected.

The role of endogenous BDNF in the periphery is unclear and may be less important (Apfel et al., 1996) although over-expression of BDNF in the skin using the keratin-14 promoter has lead to an increase of TrkB-positive neurones to 160% and reduced unmyelinated fibres to 75% of normal levels (Kirstein and Farinas, 2002).

5.2.4 NGF and peripheral inflammation

Peripheral inflammatory conditions depend on the abundant supply of NGF found within inflamed tissues, and its regulation remains a key part of the generation and probably the maintenance of persistent inflammatory hyperalgesia (McMahon et al., 1995; Dmitrieva et al., 1997; Jaggar et al., 1999; Andreev et al., 1995).

NGF is itself pro-inflammatory and can induce a sensitization of the nervous system to noxious stimuli at various levels in the message reception and transduction pathways. Peripheral sensitization of trkA positive nociceptors to thermal stimuli is dramatically increased within minutes of peripheral administration of NGF. This has been shown to have measurable effects on behavioural assays as well as electrophysiological ones. These effects have also been found to be wholly or partially reversible by sequestration of the NGF ((Lewin et al., 1994; Lewin and Barde, 1996).

NGF acts secondarily on other parts of the nociceptive sensory system as well. Importantly, NGF like BDNF is retrogradely transported by nociceptors and affects gene expression within the nociceptive cell population (Lewin and Barde, 1996). As a result, later components of the hyperalgesia, arising from either the NGF administration or its downstream peripheral inflammation and their associated changes appear to have a central component (Thompson et al., 1995).

5.3 Objectives

This study undertook to determine whether a selective ablation of BDNF in cells expressing $Na_v1.8$ using a Cre-lox system driven by a specific $Na_v1.8$ promoter could be achieved, and whether such an intervention would elicit behavioural changes in baseline and inflammatory pain models *in vivo*.

Genomic and immunohistochemical analysis was be used to demonstrate the selective loss of BDNF expression in subpopulations of DRG neurones. It was hoped that these interventions would be subtle enough to allow the development of the nervous system to continue and avoid the lethal affects which have been noted to accompany unrestricted BDNF removal.

Baseline physiological responses to non-noxious and noxious thermal and mechanical stimuli were assessed using various tests under normal and inflammatory conditions to determine any gross changes in phenotype. Motor-coordination was tested using the Rota-rod paradigm; responses to noxious mechanical, chemical and thermal stimuli were also done with Randall Sellito, intraplantar formalin and Hot-plate tests respectively. Hargreaves tests of thermal response times were collected for both unchallenged and intraplantar-NGF challenged states (a model of inflammation) to establish alterations in the development of thermal hyperalgesia, as well as Von Frey tests for mechanical threshold changes.

5.4 Materials and methods

5.4.1 Husbandry

Mice were housed in a vivarium with a normal 12-hr light: 12-hr (lights on at 07:00) dark cycle and maintained under standard condition (21 ± 1 ^{O}C, 40-50% humidity, food and water *ad libitum)*. All behavioural studies were carried out during the light cycle. All experiments were carried out on female animals between the ages of two and three months old. The 12 animals were drawn from 8 different litters to avoid any "litter effect". All tests were conducted under single blind conditions. The weight of the study animals were taken to determine if there were any obvious differences in the populations studied.

5.4.2 $Na_v1.8$-specific BDNF Knock-out mouse generation

BDNF-Floxed mice (Dr. M. Rios, Cambridge, Mass.) were genotyped to determine the gene complement. These mice were engineered to contain loxP sites around the single coding exon 5 of the BDNF gene using standard gene targeting protocols (Rios et al., 2001) (see 5.4.4). By exploiting the ability of the bacterial recombinase enzyme Cre to recognise these loxP sequences (Hamilton and Abremski, 1984) and specifically delete all genes enclosed by a pair of sites, targeted gene ablation can be achieved (see chapter 1).

This method has been successfully used in a number of studies to achieve tissue and temporally controlled gene ablation (Gu et al., 1994). In this study the use of a promoter specific for nociceptive neurones (the $Na_v1.8$ locus) allowed the generation of mice in which the BDNF gene could be removed in nociceptive neurones starting around embryonic state E-13 to 15 and continuing thereafter. This expression pattern follows the observed Cre expression pattern during development when driven by the $Na_v1.8$ promoter (Stirling et al., 2003).

In order to obtain the study population with all copies of BDNF in $Na_v1.8$ expressing cells knocked out but only one copy of the transgenic $Na_v1.8$ sequence (containing the $Na_v1.8$Cre allele), thus allowing the expression of the normal sodium channel, homozygous floxed individuals were crossed with homozygous $Na_v1.8$Cre

expressing mice (Stirling et al., 2003) to generate double heterozygous offspring. These mice were then back-crossed with BDNF homozygous mice to create the target population.

F1 generation

BDNF -/- : Cre +/+ X BDNF +/+: Cre -/-

All offspring

BDNF +/-: Cre +/-

F2 Back-cross

BDNF +/-: Cre +/- X BDNF -/- : Cre +/+

Offspring (expected ratio 1:1:1:1)

BDNF +/-: Cre +/+ Heterozygous BDNF floxed, no Cre

BDNF +/-: Cre +/- Double heterozygous animals

BDNF -/- : Cre +/+ Homozygous BDNF floxed, no Cre (Littermate controls)

BDNF -/- : Cre +/- Homozygous BDNF floxed, with Cre (test population)

The number of animals of each genotype which survived to adulthood was recorded to determine if there were any deviations from the expected ratios.

5.4.3 Genomic DNA Preparation

Tissue was collected from 0.5cm lengths taken from the tips of the tails, and placed in 700µl Tail buffer (50mM Tris-HCL, pH8.0; 100mM EDTA; 100mM NaCl; 1% SDS) containing 0.5mg/ml proteinase K (20µl) (Sigma-Aldrich, Ca.). This preparation was incubated overnight at 37^{O}C to break-down the tissue allowing the genomic DNA to be more easily harvested. 250µl of saturated NaCl (6M in dH_2O) was added to the resultant slurry and mixed by inversion. The mix was centrifuged (14K rpm, 10 min, RT) and 750µl of resultant supernatant transferred to a new Eppendorf tube. 750 µl isopropanol was added to precipitate the DNA and again centrifuged to collect the

precipitate. The resulting DNA pellet was washed in 70% ethanol, air dried and resuspended in 30µl of dH_20.

5.4.4 Genomic analysis of mice

The genomic DNA harvested from the test animals was subjected to PCR-based protocols to determine the genetic complement.

5.4.4.1 BDNF gene detection

To determine whether the normal BDNF gene or the Floxed BDNF gene was inherited a set of primers was designed (supplier; Sigma-Genosys Ltd, Pampisford, UK).

Figure 5. 3 Structure of BDNF gene

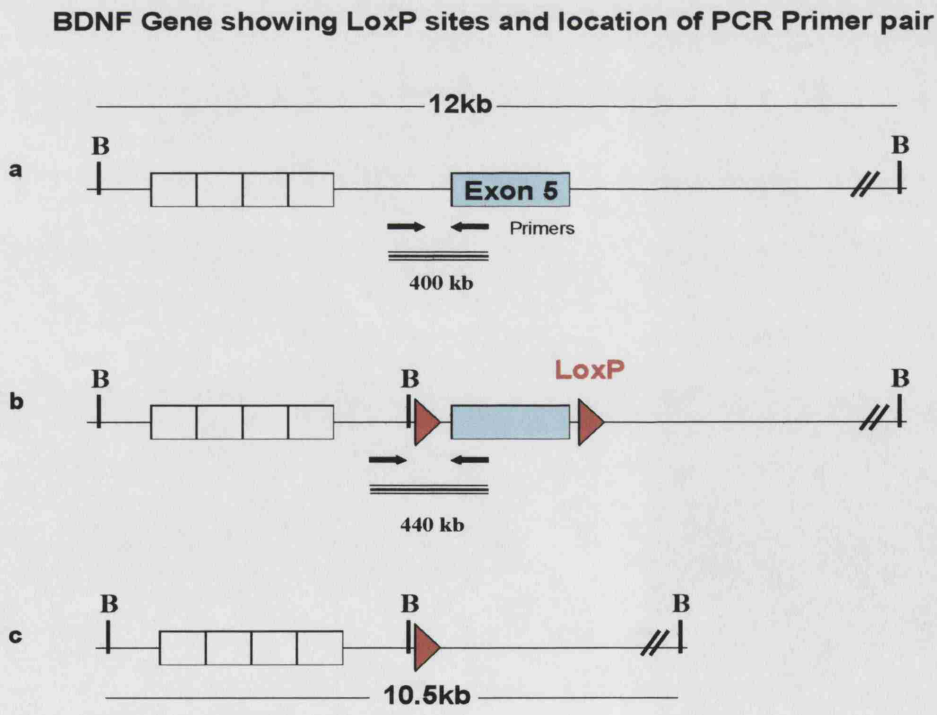


Figure 5.3: a) the wild-type allele showing the four non-coding alleles and the single coding allele (Exon 5). The location of the primer pair used to genotype animals is

indicated along with the product size. b) The Floxed transgene with the paired LoxP sites flanking the coding exon. c) The structure of the locus after the excision of exon 5 by Cre-recombinase (Rios et al., 2001). Abbreviations: B, *Bgl*II restriction site.

These PCR-primers were constructed to flank the insertion site of one of the loxP sites at the beginning of the single coding exon of BDNF, thus allowing the the non-floxed wild type allele (400bp) to be easily distinguished from the floxed BDNF allele (440bp). A BLAST search done showed no significant interaction between the chosen primers and non target genes.

Forward PCR Primer BDInI 5' TGG GAT TGT GTT TCT GGT GAC 3'
Reverse PCR Primer BD3 5' GCC TTC ATG CAA CCG AAG TAT G 3'

PCR reaction mix for one reaction (total 30µl) as follows; Taq polymerase (0.6µl); 10x PCR Buffer (Gibco, UK) (3µl); 25mM $MgCl_2$ (1.6µl); 5mM dNTP's (0.6µl) (1:1:1:1 mix of dATP, dGTP, dTTP, dCTP from Sigma, UK); 20µm Forward primer (1µl); 20µm Reverse primer (1µl); dH_20 (21.2µl); Sample DNA (1µl).

PCR Cycling conditions – PCT-220 DNA Dyad (MJ Research) Machine

1 cycle	1 x 92^{O}C	2 min
35 cycles	1 x 92^{O}C	30 s
	1 x 63.4^{O}C	30 s
	1 x 72^{O}C	30 s
1 cycle	1 x 72^{O}C	10 min

The resultant products were resolved on a 1.5% agarose (Invitrogen) gel and photographed.

5.4.4.2 Nav1.8Cre gene detection

Figure 5. 4 Structure of $Na_V1.8$ locus

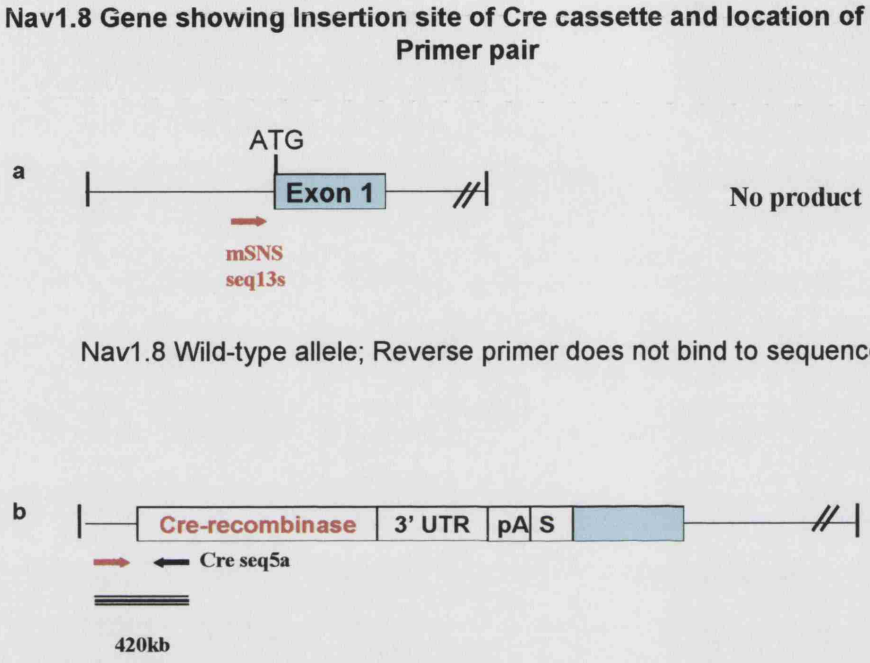


Figure 5.4: a) the wild-type allele showing the first exon and ATG start codon. The binding site of the forward primer mSNS seq13s is shown, the reverse primer does not bind. b) The Knock-in transgenic allele. The Cre-recombinase cassette contains the gene together with a 3' untranslated region (3'UTR), a polyadenylation signal (pA) followed by a stop signal (S), disrupting the beginning of the $Na_v1.8$ sequence including the ATG codon. The Diagnostic PCR product of 420kb is generated by the primer pair illustrated (Stirling et al., 2003).

The detection of the $Na_V1.8$ controlled Cre gene was carried out in a similar fashion as described for the BDNF transgene. The Cre gene was inserted into the translation start site of the first coding exon of native $Na_V1.8$ gene (Figure 5.4).In order to detect the presence of the transgenic allele two sequences were designed; Cre seq5a and mSNS seq13s. This primer pair was designed to detect only the presence of the inserted Cre

sequence and give a band of 420bp when present with no band appearing in the wild-type $Na_v1.8$ gene (Stirling et al., 2003)

Because of the difficulty of non-specific products occurring a Touchdown PCR approach was adopted (Don et al., 1991). Touchdown PCR involves decreasing the annealing temperature by 0.5-1^{O}C per successive cycle to a touchdown annealing temperature which is then used for between 10-15 cycles. It is based on the idea that any differences in Tm (Annealing temperature) between correct and incorrect annealing gives a two-fold difference in product amount per cycle, thus an enrichment of the correct product will occur to the detriment of any incorrect products.

Primers for $Na_v1.8$Cre detection

mSNS seq13s 5' TGT AGA TGG ACT GCA GAG GAT GGA 3'

Cre seq5a 5' AAA TGT TGC TGG ATA GTT TTT ACT GCC 3'

PCR Cycling conditions – PCT-220 DNA Dyad (MJ Research) Machine

1 cycle	1 x 94^{O}C	2 min
10 cycles	1 x 94^{O}C	30 s
	1 x 65^{O}C	15 s
	Decrease by 0.5^{O}C every cycle	
	1 x 72^{O}C	60 s
30 cycles	1 x 94^{O}C	30 s
	1 x 60^{O}C	15 s
	1 x 72^{O}C	60 s
1 cycle	1 x 72^{O}C	10 min

The resultant products were resolved on a 1.5% agarose (Invitrogen) gel made up in 1 X TAE buffer with added ethidium bromide for DNA detection with UV illumination and photographed.

5.4.5 Behavioural tests

5.4.5.1 Rota-rod test

Naïve mice were initially allowed to acclimatise to the stationary rod (model 7650, Ugo Basile, Italy) for 120s. After this acclimatisation period, animals were placed on the stationary rod and 5 seconds later the rod was rotated at a constant speed of 20rpm for 300 seconds and the time taken for the animal to either fall off or rotate passively recorded. On subsequent days three further trials were done in a similar manner as above but with the rod accelerating from 20 rpm to the maximum of 40 rpm within the 5 minute test period. The time that each mouse was able to stay on the rod without either falling or passively rotating was recorded. The mean ± SEM was determined and analysed with a Student's t-test.

5.4.5.2 Randall Sellito test

This test was performed by a modification of the method usually employed on rats where the paw is placed between a blunted Perspex point and a flat surface and subjected to increasing force until the animal flinches or removes the paw. In the case of the mice under investigation this method proved problematic due to the small size of the paw and the high degree of stress induced in the animal, therefore a system of passively restraining the animal in a Perspex tube allowing the tail to be used to measure the latency period was employed. After a 15-minute acclimatization period, the area of the tail approximately 1cm from the base was subjected to an increasing pressure and the force (grams) at which a response was elicited noted. This was repeated three times and the mean ± SEM calculated and assessed by means of a Students t-test.

5.4.5.3 Formalin test

Subcutaneous injection of dilute formalin (15µl, 5% dilution of stock formalin (40%w/v) in saline) was used as a model of acute peripheral injury. On the day of the testing the two groups of animals (n=6 each group) were singly housed in a Perspex box

and given 30min to habituate to the testing environment. Animals were then placed in a Perspex tube-restrainer and the left hind paw injected with formalin. Nociceptive behaviour was taken to be licking and biting the injected paw only. The time that these two activities were displayed by the animal recorded in 5 minute bins until 60 min had passed. Differences between the two groups were assessed with two-way ANOVA followed by *post-hoc* Tukey tests. All animals were euthanased immediately following completion of this test.

5.4.5.4 Hot-Plate test

The testing of the response to thermal stimuli was done using a hot plate analgesia meter (Ugo Basile, Italy). The animals were habituated for the equipment for 15 min before the basal metal plate was heated to the test temperature and the animal returned to the environment. The time taken for a response was recorded. Lifting of any paw, biting and licking, rearing up on hind legs, paw flinching or jumping was taken as the cut-off point with a maximum of 60 seconds for temperatures under 52.5 ± 0.5 ^{O}C and 30 seconds above 53 ± 0.5 ^{O}C. One test was conducted per animal per day. Four temperatures were assessed on subsequent days with each test group having 12 animals. The means for each temperature ± SEM was determined and subjected to a two-way ANOVA test of significance followed by *post-hoc* Tukey test.

5.4.5.5 Von-Frey test of mechanical thresholds

Von-Frey hairs were used to assess the mechanical withdrawal threshold on the plantar surface of the hind paw. After acclimatisation to the testing environment (30 min), that consists of a Perspex box with a meshed base, mechanical sensory thresholds were determined by paw withdrawal to application of a series of von Frey filaments to the glabrous surface of the hind paws. Calibrated von Frey filaments were applied five times per paw with enough force to cause buckling of the filament. Eight filaments were tested in ascending order from 0.219g to 7.59g. The percentage response for each filament was determined by scoring the positive responses (both supraspinal; biting, licking, and simple hyper-reflexia; paw lifting); i.e. number of trials accompanied by a response /5 x 100%.

Left and right hind paws were tested with 3 minute breaks between subsequent tests. Each group contained 12 animals and the mean ± SEM was assessed using two-way ANOVA with post-hoc Tukey tests.

5.4.5.6 Hargreaves test of thermal nociceptive thresholds

The Hargreaves method (Hargreaves et al., 1998)was used to measure thermal hyperalgesia using the plantar test (Ugo Basile, Comerio, Italy). Mice were habituated for 15-30 minutes to the apparatus which consists of Perspex observation chambers into which the animals are placed, upon a glass table. A mobile radiant heat source was located under the table and focused onto the rear paw midway between the paw edges level with the base of the thumb joint. Paw withdrawal latencies (PWL) were taken three times for both hind paws with at least 5-minute intervals between each subsequent test. The mean of the three measures represented the latency of paw withdrawal and was taken as a measure of thermal pain responses. The equipment was calibrated to give a mean PWL of approximately 10 seconds.

5.4.5.7 Nerve Growth Factor (NGF) induced thermal hyperalgesia

In order to test the effect of the $Na_v1.8$-specific knock-down of BDNF on the development of NGF induced thermal hyperalgesia, animals were injected with 50ng HrNGF (5µl carrier volume) (Human recombinant Nerve Growth Factor 4.2mg/ml in 20mM succinate buffer diluted to 10µg/ml with saline) into the subcutaneous plantar surface of the left hind paw. The injection was done under anaesthesia induced by 4% halothane/oxygen to ensure reproducible injections of a consistent position and depth. The animals recovered within 3 minutes. The development of the thermal hyperalgesia was tracked for various time points after the injection up to a maximum of 24 hours. Two-way ANOVA was carried out followed by *post-hoc* Tukey analysis.

5.4.6 Statistical analysis

All data are presented as mean ± SEM. The statistical significance between groups were assessed with Two-way ANOVAs followed by Tukey multiple comparison tests or Student's unpaired t-tests where appropriate. $P < 0.05$ was regarded as significant (Sigmastat 2.01).

5.5 Results

5.5.1 Survival rate of test-cross

Assessment of the genotype ratios gave the expected 1; 1; 1; 1 ratio for the four genotypes generated from the breeding program. (Figure 5.5)

Number of animals	Genotype	
49	**BDNF +/-: Cre +/+**	Heterozygous BDNF floxed, no Cre
52	**BDNF +/-: Cre +/-**	Double heterozygous animals
59	**BDNF -/- : Cre +/+**	Homozygous BDNF floxed, no Cre
57	**BDNF -/- : Cre +/-**	Homozygous BDNF floxed, with Cre

This result indicated that the deletion of BDNF specifically in $Na_v1.8$ expressing cells (almost entirely sensory neurones in the peripheral nervous system - Sterling et al) did not affect the viability of the animals. Furthermore, the weights of the two groups of females (8 – 10 weeks post natal) were not significantly different. $Na_v1.8$-specific BDNF knock-out (KO) 16.88 ±0.88g compared with wild-type (WT) littermates 17.72 ± 0.42g ($p = 0.43$, $n = 6$; unpaired, heteroscedastic student's t-test).

5.5.2 Motor coordination

Measurements of the motor co-ordination of the knock-out group by means of the Rota-rod test (Figure 5.6) also failed to show any significant differences. All animals (n=12) in both groups were able to stay on the rod turning at a constant speed of 20 rpm for the maximum time of 300 seconds. Subjecting the animals to the increasing speed test also failed to highlight any differences in the two groups, BDNF KO 140.1 ± 19.6 s; control WT Littermates157.0 ± 14.5 s ($p = 0.43$, $n = 12$; unpaired, heteroscedastic student's t-test).

5.5.3 Mechanical force

The Randall-Sellito test (Figure 5.7), which measures nociceptive responses to mechanical squash, was applied to the tails of 9 animals in each genotype. The means obtained were BDNF KO 68.3 ± 4.3 g; control WT 71.6 ± 8.3g. There was no significant difference noted in the responses ($p = 0.74$, $n = 9$; unpaired, heteroscedastic student's t-test).

5.5.4 Supraspinally mediated thermal hyperalgesia

To assess hyperalgesia or analgesia, a hot-plate analgesia meter was used to activate thermal nociceptors. This method (Woolfe and MacDonald, 1994) isolates the responses due to the supraspinal activity. The mean outcome for four trials at 47.5 ± 0.5; 50 ± 0.5; 52.5 ± 0.5 and 55 ± 0.5 ^{O}C were plotted (Figure 5.8). Two way ANOVA analysis followed by post-hoc Tukey repeated tests showed that the difference in the mean values among the different trial temperatures is greater than expected by chance after allowing for the effects of temperature, with the BDNF KO group being more sensitive to the stimuli than the control WT group. There is a highly statistically significant difference ($p < 0.001$). *Post-hoc* tests showed that this difference was observable at all the temperatures tested.

5.5.5 Allodynia and mechanical sensitivity

Comparisons between the responses to mechanical stimulation from various von Frey hairs (Figure 5.9), showed that there was a significant difference between the response curves obtained, with the BDNF KO group showing allodynia compared to the control WT group ($p = 0.023$, $n = 12$ two-way ANOVA). Tukey analysis showed that this difference is entirely attributable to the responses to the lower-weight fibres (under 0.6g) ($p <= 0.01$); there being no statistical difference in the responses of the two groups to von Frey fibres of 1g or heavier (Figure 5.10).

5.5.6 Hyperalgesic effects of subcutaneous NGF *in vivo*

The injection of 5μl of carrier solution containing 50ng Hr-NGF created a small pocket under the subcutaneous plantar surface of the left hind paw, which disappeared within five minutes. The dose of 50ng NGF/paw (2.5ng per gram weight) elicited a marked thermal hyperalgesia in the ipsilateral but not contralateral paw, which persisted until the end of the experiment 24 hrs later (Figure 5.11). In agreement with earlier findings in rats (chapter three) there appeared to be a biphasic response but this was less obvious than in the rat (Figure 3.5.) .An acute phase of hyperalgesia beginning at least 30 minutes into the testing period (probably much sooner) with some recovery at 1 hour p.i. followed by a second period of hyperalgesia which appeared by 2 hours which was resolved 24 hours later.

There was a highly significant response between the two groups ($p<0.001$; $n = 6$ two-way ANOVA). The $Na_V1.8$ specific BDNF KO animals showed a markedly reduced intensity in the NGF-induced thermal hyperalgesia when compared to the littermate control group (Figure 5.11). However, neither the first nor the second phase of the response was abolished, both being reduced in the knock-out animals.

5.5.7 Tonic response to intraplantar formalin

The biphasic response to intraplantar formalin was reproduced in mice in agreement with reported responses in the literature (Hunskaar et al., 1985; South et al., 2003). The first phase (0-10min) showed no significant difference between the two groups (Figure 5.12) but the second phase was reduced approximately 2.5-fold in the knock-out mice. Further investigation showed that this reduction in the pain responses evoked in the second phase of the formalin test occurred mainly 30 - 40 minutes after intraplantar injection of the formalin solution in the hind paw (figure 5.13).

Figure 5. 5 BDNF and Nav1.8Cre genomic analysis

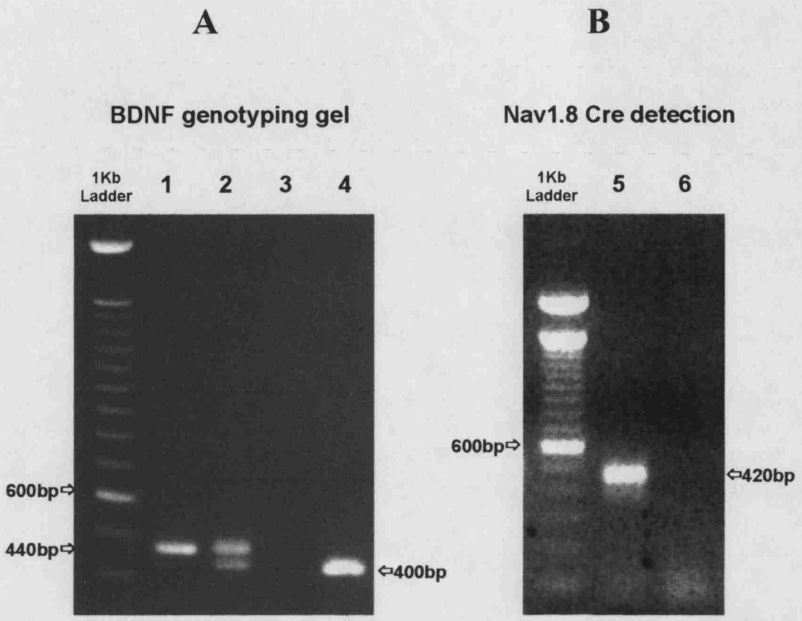


Figure 5.5; 1.5% Agarose gels showing the expected bands from the genotyping of the resultant offspring. A) BDNF gel: Homozygous BDNF floxed -/- (440bp band only), lane 1; Heterozygous +/-, lane 2 (BDNF floxed band [440bp], and WT band [400bp]); Negative control, lane 3; Homozygous wt +/+ (400bp band only), lane 4;

B) $Na_V1.8$cre gel: positive (420bp band), lane 5; Wildtype (no band), lane 6

Figure 5. 6 Rota-rod test

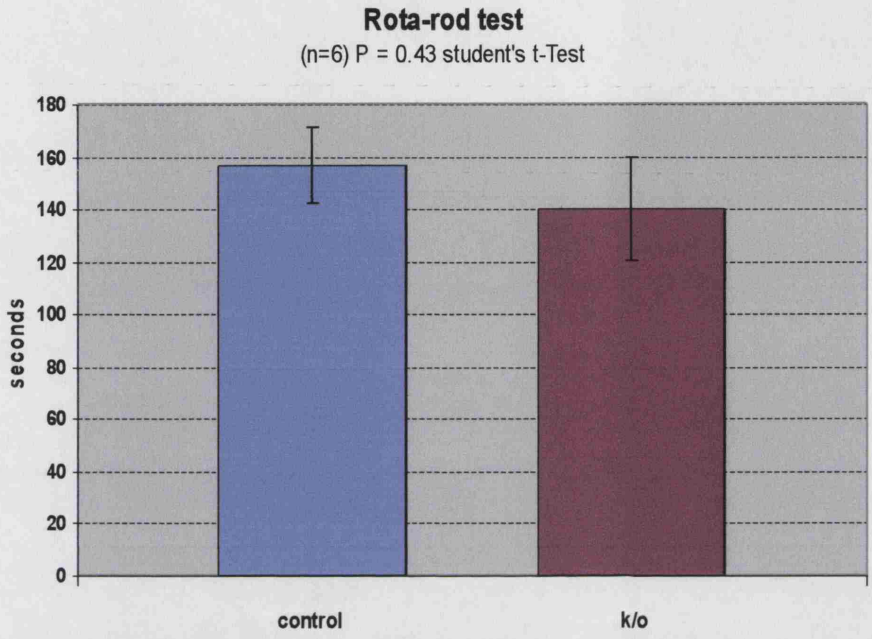


Figure 5.6; Motor coordination of $Na_v1.8$-selective BDNF knock-down transgenic mice compared to control littermates (materials and methods 5.4.5.1). No significant differences were noted (n = 6).

Figure 5. 7 Randall Selitto test

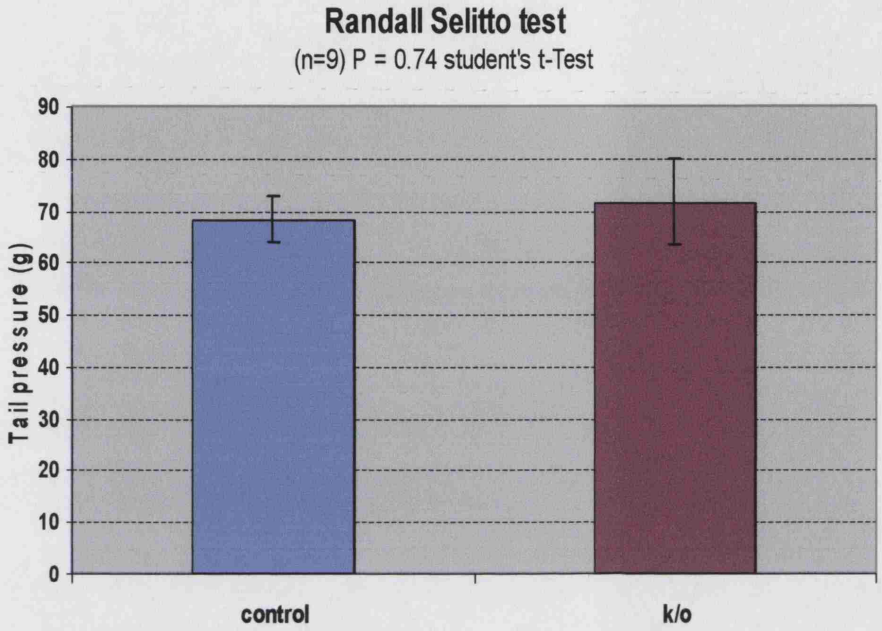


Figure 5.7; The response thresholds of $Na_V1.8$-selective BDNF knock-out transgenics to pressure thresholds on their tails (methods 5.4.5.2) is not significantly different to the thresholds of control littermates (n = 9).

Figure 5. 8 Hot-plate test

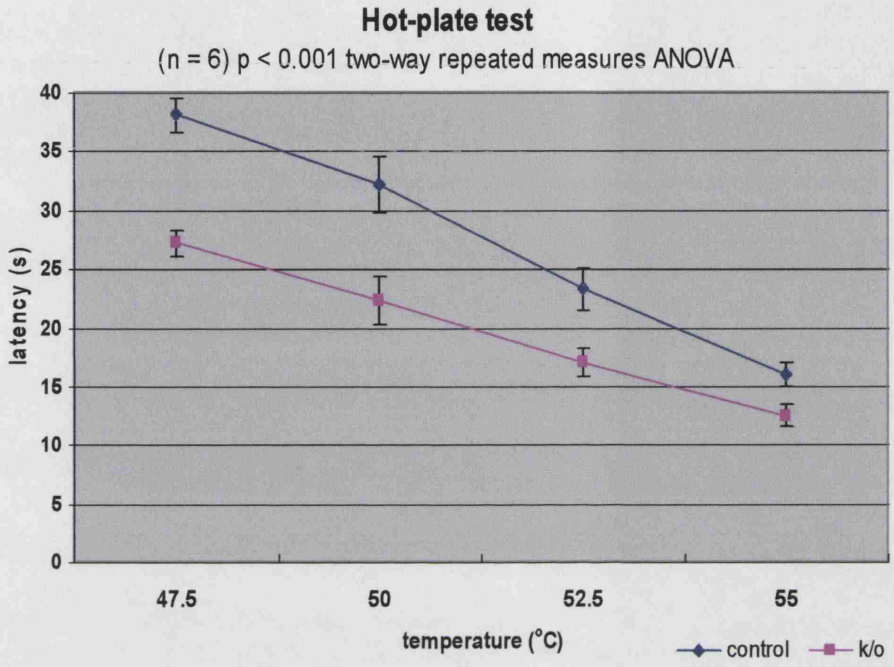


Figure 5.8; The $Na_V1.8$-selective BDNF ablation resulted in an increase in thermal sensitivity as assessed by the hot plate analgesia-meter compared to control littermates.

Figure 5. 9 Von-Frey test

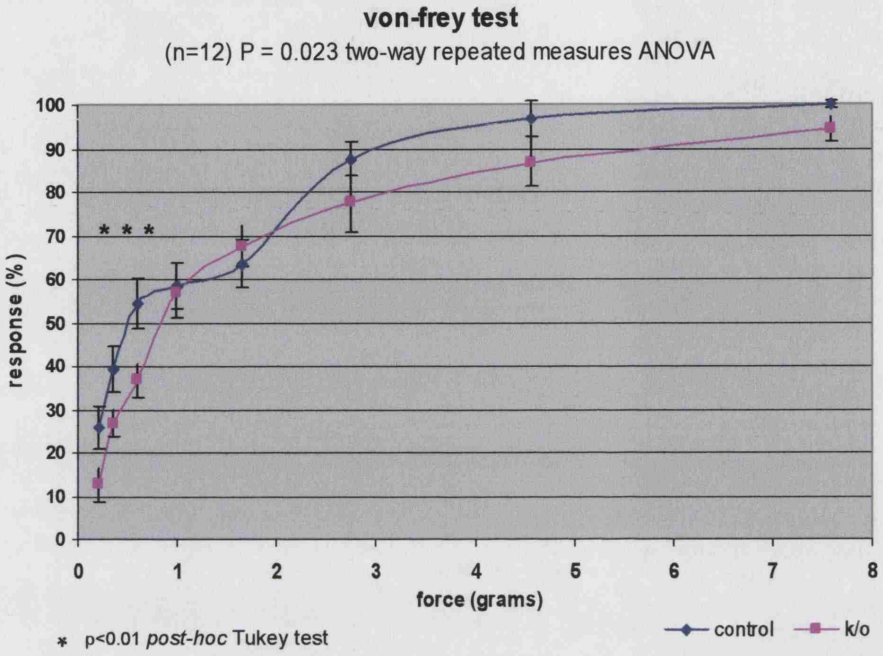


Figure 5.9 a: Response curves of BDNF KO and WT mice to a range of von Frey hairs ranging from non-noxious (under 1 gram) to noxious (over 1 gram). The BDNF KO mice show a significantly reduced sensitivity to non-noxious stimuli compared to the WT group ($p<0.05$). At noxious stimulation level (hair weights over 1 gram) there is no difference in the response curve between the two groups.

Figure 5. 10 Von-Frey test (50% threshold)

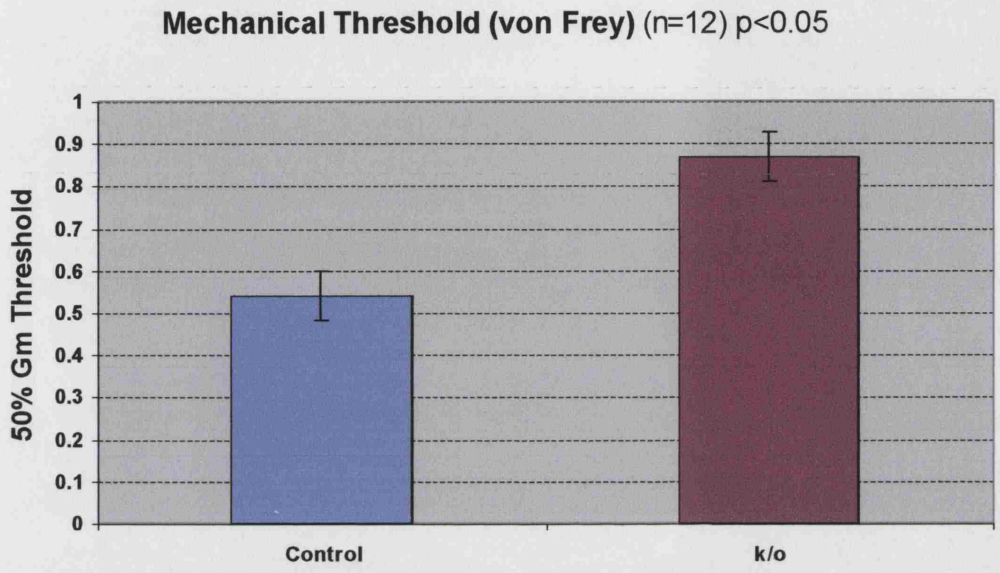


Figure 5.10; 50% threshold value of BDNF KO mice compared to WT littermate controls. The KO mice have a significantly increased threshold compared to WT littermate co

Figure 5. 11 Hargreaves' test of thermal hyperalgesia

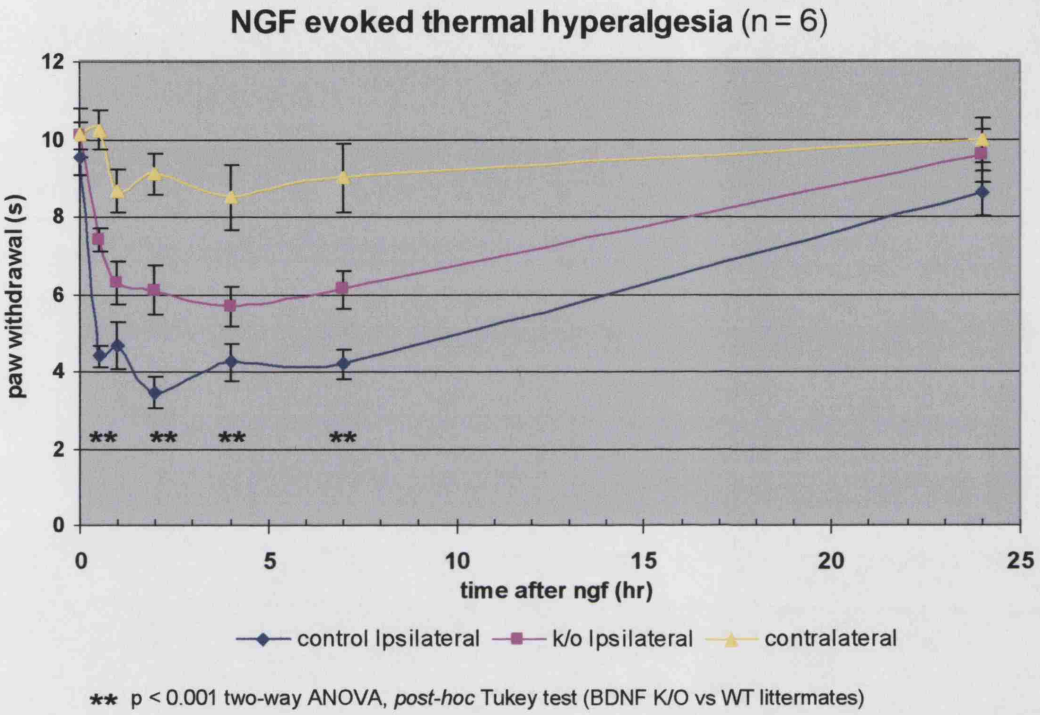


Figure 5.11; development of thermal hyperalgesia after intraplantar treatment with human-recombinant NGF (at t = 0) in the ipsilateral paw of BDNF KO or WT control animals. The development of a biphasic thermal hyperalgesia is displayed in the ipsilateral but not contralateral paw. This effect is significantly reduced in the KO group compared to the control in both the initial (up to 1 hr) and second phases (post 1 hr). Note that the baseline response (shown at t = 0) to a thermal stimulus resulting in a reflex pathway activation (Hargreaves' type) is the same in both the BDNF KO and WT groups. This indicates that C-fibre derived BDNF is not implicated in this signal pathway.

Figure 5. 12 Formalin test, acute vs chronic phases

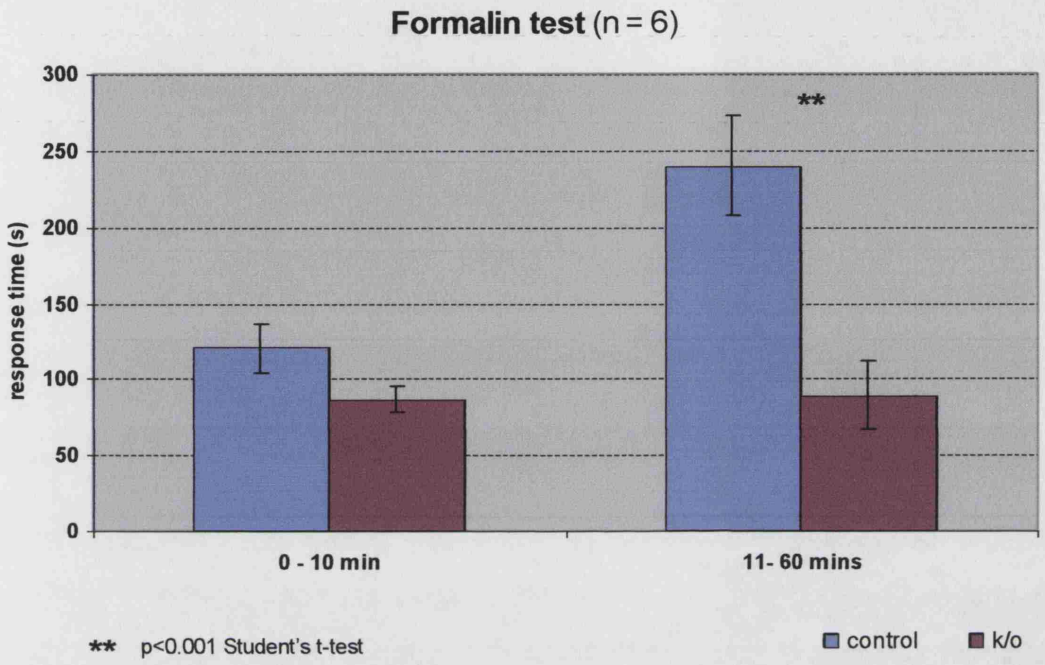


Figure 5.12; Formalin-induced injury behaviour (Phases). Intraplantar injection of formalin results in licking and biting activity that can be divided into two phases. Phase 1 encompasses the first 10 min post injection and phase 2 begins at 10min p.i. continuing until 60 minutes after injection. The spatiotemporal knockout of BDNF in the $Na_V1.8$ positive-sensory neurones of mice significantly decreases the painful response to an injury-inducing stimulus measured during phase 2 of the formalin test approximately 2.5-fold, whilst leaving the phase 1 response unaffected.

Figure 5. 13 Formalin test; development

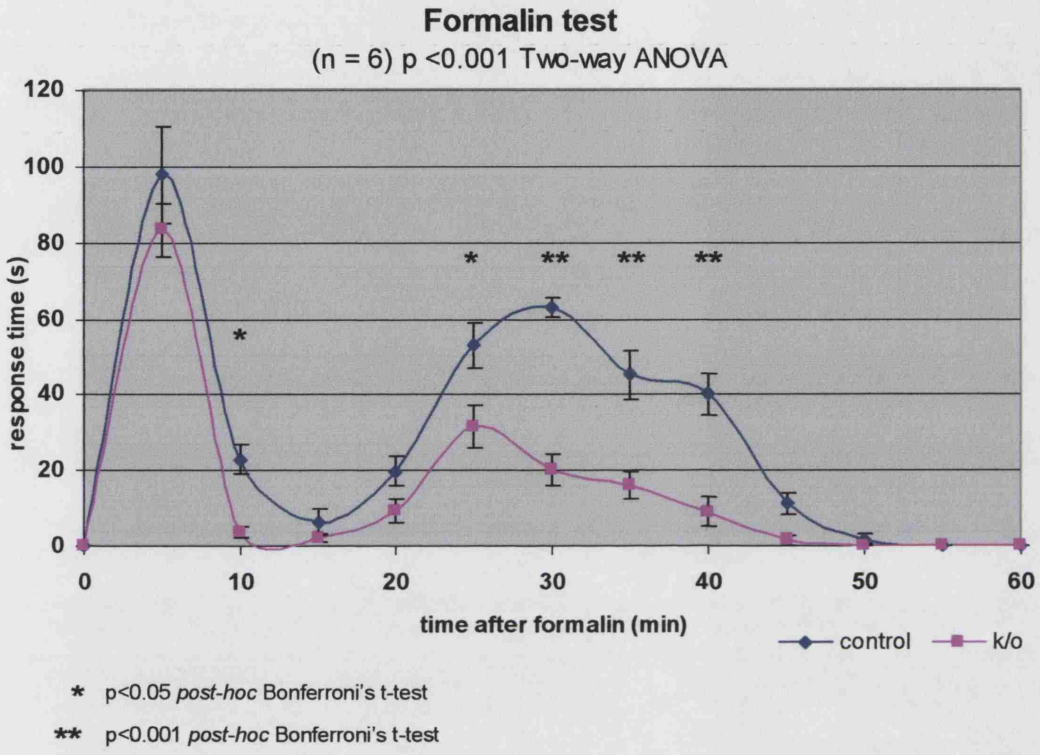


Figure 5.13; Formalin-induced injury behaviour (Time course). Graph showing the total time spent engaged by $Na_V1.8$-specific BDNF KO or WT mice in biting and licking of the injected hind paw per 5 minute periods up to 60 minutes p.i. $Na_V1.8$-specific BDNF KO mice show a diminished pain response at 25-40 min after formalin ($p<0.001$) as well as at 10 min p.i ($p<0.05$).

Figure 5. 14 BDNF expression in DRG; BDNF KO vs WT

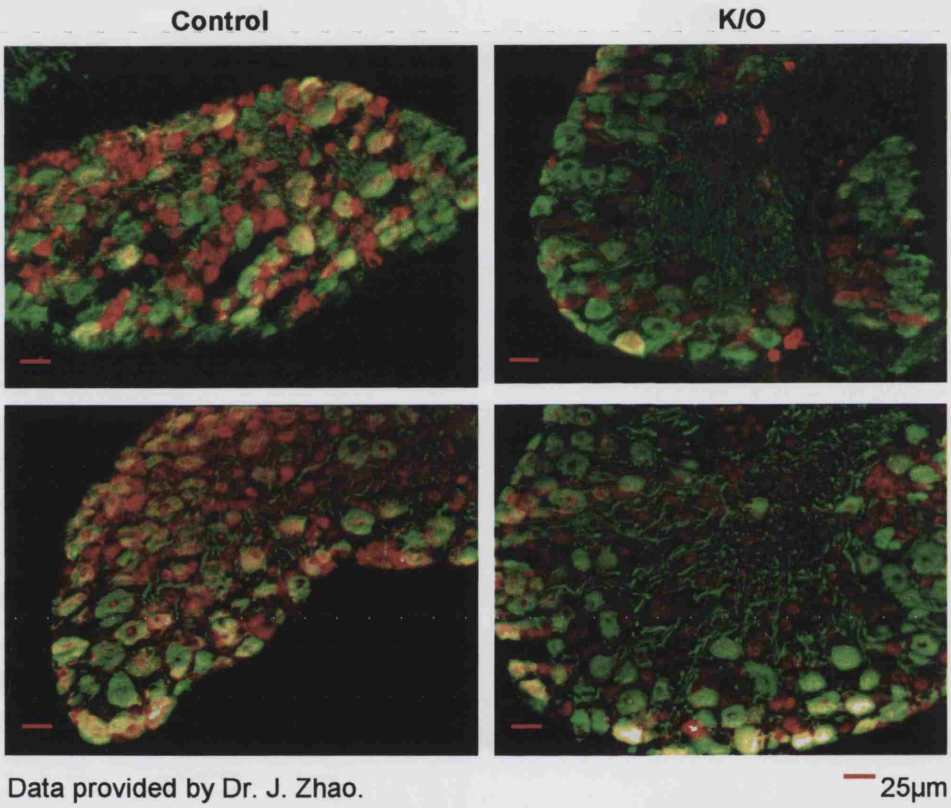


Figure 5.14; Immunohistochemical analysis of BDNF expression patterns of Lumbar 4/5 DRG from both $Na_v1.8$-specific BDNF K/O mice and from wild-type littermate controls. The two right DRG sections from the KO animals display less BDNF immunoreactivity (shown in red) than compared with the control DRG sections (left). These sections have also been counterstained for the presence of the large diameter marker N52 (green). In the conditional KO animals there is continued expression of BDNF in a small number of predominantly large-diameter N52-positive neurones.

5.6 Discussion

5.6.1 $Na_V1.8$-specific BDNF ablated mice; survival and characteristics

The survival of the BDNF total knock-out mouse only up to two weeks post-natally (Jones et al., 1994; Ernfors et al., 1994) has set-back our understanding of the role of BDNF in the adult nervous system and specifically its role in nociceptive transmission pathways of physiological or acute pain and pain from noxious and non-noxious stimuli in inflammatory and neuropathic situations.

In order to address this question the BDNF gene was specifically ablated in a new strain of mice created by crossing a BDNF floxed strain (Rios et al., 2001) with a strain expressing Cre-recombinase under the control of the voltage-gated sodium channel 1.8 ($Na_v1.8$) promoter (Stirling et al., 2003). $Na_v1.8$ is expressed only in sensory neurones which are made up primarily of unmyelinated C-fibre possessing small neurones and thinly-myelinated A-fibre neurones (Akopian et al., 1999; Benn et al., 2001; Djouhri et al., 2003; Renganathan et al., 2000). Both of these populations transmit nociceptive information (Okuse et al., 1997; McCleskey and Gold, 1999). 18% of large-diameter (>25μm), well-myelinated neurones, as identified by NF200 staining (Benn et al., 2001), express $Na_v1.8$. A subset of these large cells will also express the receptor for BDNF and may be subject to loss in any BDNF knockout mouse produced. The small diameter population of cells which can be divided into two categories, peptidergic or non-peptidergic also express $Na_v1.8$ (42% and 40% respectively) (Benn et al., 2001). These populations require NGF and GDNF respectively and may be unaffected by the knockout of BDNF.

The offspring which resulted from this cross survived postnatally well into adulthood (over three months) with no apparent changes in gross morphology (growth rates identical as determined by weight at three weeks post-natal measurements; WT control 17.7 ± 0.5g vs BDNF conditional KO 16.9 ± 0.9g) or mortality rates compared with wild-type littermates, all genotypes generated were distributed according to normal Mendelian ratios (BDNF. -/- with $Na_V1.8$Cre +/-; BDNF -/- with $Na_V1.8$Cre +/+; BDNF +/- with $Na_V1.8$Cre +/- and BDNF +/- with $Na_V1.8$Cre +/+).

All genotypes also survived well past the third month after birth, a time-point when all the BDNF homozygous unconditional KO animals had died (Heppenstall and Lewin, 2001). Additionally, there were no abnormal home cage behaviours noted as evidenced by bald patches, absence of whiskers etc (Crawley and Paylor, 1997).

5.6.2 BDNF requirement by adult DRG neurones

It has been shown however that the cells which express BDNF do not generally express the TrkB receptor in the normal situation (Huber et al., 2000) and are therefore less likely to require endogenous BDNF for support. The subpopulation expressing the TrkB receptor is the myelinated Aδ-fibres which do not normally participate in nociceptive information relay circuits (Lawson, 2002).

Data from Heppenstall et al (2001) demonstrated no difference in IB4-positive and CGRP expressing populations of lumbar region 4/5 DRG neurones in the P7 stage of the BDNF total knock-out mice (Heppenstall and Lewin, 2001) and supports our hypothesis that BDNF ablation after E13 may not have overt developmental consequences. Further work to characterise the subpopulations in adult DRG as well as peripheral nerve innervation arbours in homozygous conditional KO animals compared to control littermates will be needed to confirm this.

Additional data to support the escape of developmental anomalies in this cross comes from earlier *in vitro* work on cultures of DRG or nodose ganglia sensory neurones from BDNF total knockout animals (Huber et al., 2000). These cultures displayed TrkB expression and early sensory neuron survival which was independent of endogenous BDNF, i.e. there is no BDNF autocrine loop requirement in neurogenesis.

Another study showed that the cells which eventually respond to BDNF in adult life (the TrkB population), start out their development requiring NT-4 for their survival during early gangliogenesis and then switch to BDNF on maturity (Liebl et al., 2000). This study also reported that when the developing ganglia is deprived on NT-4 the BDNF responsive population becomes NT-3 responsive and provide proprioceptive neurones instead of BDNF-dependant mechanoreceptors (Aδ-fibres). The source of the BDNF to support this subpopulation during adulthood was not demonstrated however, and the possibility that this is unaffected in this conditional BDNF KO strain remains.

During later gangliogenesis and around birth there seems to be evidence for transitory requirements of BDNF by certain populations of DRG neurones, such that there is a loss of up to 36% of neurones in the homozygous BDNF knockout mice (Ernfors et al., 1994; Liebl et al., 1997) has been reported, leading eventually to the early death of these mice. These figures are in agreement with counts of adult neurones in DRG which are TrkB positive (Kirstein and Farinas, 2002). This population of cells may therefore obtain their required BDNF from a non-$Na_V1.8$ expressing cell population. There were also no abnormally early deaths observed in our conditional KO mice group providing further indications that these neurones remain unaffected.

Taken together, these findings support our preliminary conclusion that the $Na_v1.8$-specific knockout of BDNF has a minimal affect on the development of the nociceptive population of cells.

Immunohistochemical analysis (kindly provided by Dr. J. Zhao) has demonstrated a loss of BDNF protein in a large proportion of DRG neurones the majority of which are of the small-diameter type (Figure 5.14). Additional data to illustrate the reduction in BDNF protein in the dorsal horn both in the unchallenged and inflammatory state will support the findings contained within this chapter. Further work to characterise various neuronal subpopulations needs to be carried out to determine if there is a subtle shift in adult neurotrophin requirements as a result of this intervention which may be contributing to the phenotype observed.

5.6.3 Behavioural studies

The KO mice generated were then subjected to a variety of tests to determine the involvement in BDNF derived from $Na_v1.8$ expressing neurones in the baseline nociceptive threshold determination and also any involvement for this BDNF in inflammatory pain states. The control group was drawn from the litter mates which did not inherit the $Na_V1.8$–Cre allele and thereforecontinue to express BDNF normally. This group had therefore almost identical genetic backgrounds allowing a control to be made against differences arising from widespread allelic differences in the genetic background (strain effect) (Mogil and McCarson, 2000).

To determine the response of the $Na_v1.8$-specific BDNF KO mice (KO) to a test of motor skills (non-noxious stimuli) compared to WT control group, the rota-rod test was used. There was no significant difference obtained between the two groups indicating that the KO mice display normal motor coordination and balance. This result demonstrated that BDNF released from small-diameter neurones does not play a role in the pathways important in maintaining motor coordination. This finding supports earlier work by Jones *et al* who demonstrated that motor neuron development was not affected in the global BDNF knockout mice (Jones et al., 1994).

Other studies which showed coordination problems in BDNF ablated mice (Ernfors et al., 1994), were subsequently explained by defects in the development of the hair cells in the inner ear (Lewin and Barde, 1996) and not due to motor neuron deficits. In this $Na_v1.8$-specific BDNF knock-out mouse, the hair cells should not be affected as $Na_v1.8$ is not expressed in auditory neuronal populations and hence there would be no deletion of the gene in these cells. This accounts for the normal phenotype observed.

5.6.4 Physiological pain (acute pain)

The response of the KO mice to normal physiological or acute pain stimuli was assessed. Physiological pain has a high-threshold, is well localised and transient and has a high stimulus-response relationship. Both centrally mediated acute responses and reflex responses to acute noxious stimuli were examined using thermal, mechanical and chemical noxious stimuli. These stimuli are conducted by the nociceptors subset of sensory neurones (C and Aδ-fibres) which do not conduct innocuous stimuli under normal conditions.

The following tests were carried out to determine what role if any BDNF derived from $Na_v1.8$ positive neurones had in establishing the response to;

a) A noxious thermal stimulus leading to a spinal reflex action – Hargreaves' test,

b) A noxious mechanical stimulus leading to a spinal reflex response – Randall-Sellito test,

c) A noxious thermal stimulus involving a supra-spinal (centrally mediated) response – Hot-Plate test,

d) A noxious mechanical stimulus involving a centrally mediated response – von Frey's test

e) A noxious chemical stimulus involving action at terminal chemical receptors – First phase of the Formalin test.

The $Na_v1.8$-specific deletion of BDNF does not appear to have any effect on reflex pathways of nociceptive information processing. This was evidenced by the normal behaviour of the KO compared to WT in both thermally (Hargreaves test (a)) and mechanically (Randall-Sellito (b)) mediated responses.

In contrast however, acute noxious stimuli which lead to involvement of central processing in their relay pathways showed significant differences. This result implicates BDNF in the mediation of these responses at a spinal level.

When a thermal stimulus was applied (Hot-plate test (c)) to both the KO or WT groups the response of the KO group was found to be significantly increased at the temperatures tested. This result may indicate that the firing patterns evoked from such a stimulus may act to activate GABAergic inhibitory pathways in the dorsal horn and hence increase the latency time to such a stimulus. The removal of BDNF therefore reduces the normal latency period and leads to an increased response.

In contrast, Groth and Aanonsen reported that intrathecal administration of BDNF produces a thermal hyperalgesia (Groth and Aanonsen, 2002). It was expected that the deletion carried out would induce a hypoalgesic condition, which was not observed. However, it is of interest that administration of BDNF at both low (0.75 pMol) and high (750 pMol) concentrations the response was the same, leading to a U-Shaped response curve (Groth and Aanonsen, 2002). The possibility of an anti-nociceptive effect of endogenous BDNF could therefore not be ruled out entirely. The results of their study seems to lend support to the idea that BDNF present in the dorsal horn facilitates GABA release in a depolarisation-induced manner (Pezet et al., 2002a).

In tests of responses to mechanical stimulation involving a central response (von-Frey's test d), the KO animals again differed significantly in their latency times compared with the control group with the 50% threshold value being significantly elevated compared to the WT group (figure 5.9b). The difference was found to lie in the responses of the groups to low threshold stimuli (under 1g force). At stronger intensity von Frey hairs (over 1g) the response was identical. This suggests that there is a group of

mechanosensitive fibres which are responsible for the relay of low threshold mechanical stimuli. It is this group of fibres which may be affected by the removal of BDNF from $Na_V1.8$ positive cells. A report by Carroll et al showed that BDNF regulated the function of one class of cutaneous mechanoreceptors, independent of any survival-promoting effects on these cells (Carroll et al., 1998).

Alternatively the removal of nociceptor-derived BDNF after E13 may have led to confounding developmental changes involving the axonal targeting of mechanosensors or led to a alteration in the response dynamics of this population. Recent work has demonstrated that developing retinal ganglion cells are exposed to two sources of BDNF. One source comes from the developing retina (peripheral BDNF) and the other from the optic tectum (centrally derived source). These two BDNF sources act in concert to mediate the developing arbour and final innervation (Lom et al., 2002). A similar mechanism may be in operation in the PNS with BDNF released centrally and peripherally by developing neurones.

Presently it is thought that the adult cutaneous mechanoreceptor fibres can be classed into four groups based on their conduction velocities and response thresholds. (review (Snider, 1998)). Studies on BDNF knock-out mice soon after birth, showed that slowly adapting mechanoreceptors (SAM) that teminate in merkel end-organs and signal steady pressure, were reduced in sensitivity (but not number) about ten-fold. Rapidly adapting mechanoreceptors (RAM) on the other hand were unaffected and continue to efficiently conduct nociceptive mechanical stimuli (Carroll et al., 1998).

The first phase of the formalin test (0 –10 minutes p.i.) represents the expression of spontaneous pain behaviour in response to an immediate, short-lasting effect of the formalin irritant. This phase follows the direct activation of chemical receptors in the periphery (primary afferent fibres) leading to nociceptive firing of primary sensory neurones and does not involve central effects or inflammatory mediators (Tjolsen et al., 1992; Puig and Sorkin, 1995; McCall et al., 1996; Corderre and Melzack, 1992). This phase of the test was not affected by the knockdown of BDNF in $Na_V1.8$ positive sensory neurones until the end on the testing period (10min time point). At this time the response of the BDNF KO group was slightly lower than the WT control group. This indicated that BDNF does not play a role in the peripherally mediated action of noxious chemical stimuli.

5.6.5 Inflammatory pain

The second phase of the formalin test (10 – 60 min p.i.) constitutes the pain-behaviour resulting from a combination of ongoing sensory input and central sensitization effects and inflammatory mediators such as prostaglandins (Hunskaar and Hole, 1987; Elliot et al., 1995; Bon et al., 2002). This was substantially decreased in the BDNF KO group compared to the WT littermate controls. This data suggests that the contribution of BDNF to the establishment of central sensitization and /or inflammatory-mediated changes in the periphery is important. Removal of the BDNF expressed by small diameter $Na_V1.8$ positive nociceptors is sufficient to significantly ablate the pain-associated behaviour which accompanies exposure to formalin, especially between 20 and 40 minutes p.i. The possibility that this effect is NMDA-related was not investigated however, and the exact mechanism needs to be clarified. Recent studies have increased the evidence for relative contribution of NMDA and NK1 (neurokinin) receptors to second phase pain behaviour compared to inflammatory effects of ongoing peripheral input ((Puig and Sorkin, 1995; Shimoyama et al., 1997; South et al., 2003) has increased. This suggests that BDNF may be acting though the dorsal horn NMDA pathway as well.

Kerr et al (1999), presented data related to the performance of mice in the formalin test after application of a trk-B-IgG molecule which removed the endogenous BDNF from the dorsal horn. In the paper they demonstrated that BDNF knock-down affects the second phase of the formalin phase in a similar manner as demonstrated above (Kerr et al., 1999). However, two methodological complications were apparent in this study preventing a meaningful comparison with the data presented within. Firstly, the TrkB-IgG scavenging molecule employed was not shown to remove BDNF selectively and may well have removed NT4, the other neurotrophin with moderate affinity for the TrkB receptor. Secondly, the animals were pre-treated with NGF which is known to upregulate BDNF levels and therefore the data does not represent the *in vivo* situation accurately. The present study tries to answer the question of BDNF involvement in the second phase of the formalin evoked response in a more parsimonious manner.

The contribution of endogenous BDNF to inflammatory pain evoked by peripheral NGF application was also tested. The thermal hyperalgesia of the mice was tested using a radiant heat source (Hargreaves test). The knock-down of BDNF in $Na_V1.8$ positive

neurones, significantly reduced the development of both the early and secondary phases of thermal hyperalgesia associated with the intraplantar injection of NGF.

The production of NGF in inflamed tissues made available through cell damage and mast-cell degranulation plays an important role in the inflammatory process. Recent evidence has shown that NGF produced during inflammation has multiple effects on the sensory system acting both peripherally and centrally after retrograde transport up the axons of TrkA positive neurones (Lewin et al., 1994). BDNF is expressed in this population of neurones under normal conditions and after an inflammatory insult nearly all of the trkA positive C-fibre bearing neurones express BDNF. Additionally, a subset of both IB4 positive (non-peptidergic) C-fibres as well a subset of myelinated A-fibres begin to express BDNF de novo although both groups are very small in comparison to the CGRP-positive TrkA expressing C-fibre population. This sequence of events indicates that BDNF regulation has a role to play in mediating the inflammatory response (McMahon et al., 1995; Cho et al., 1997a; Michael et al., 1997).

The initial thermal hyperalgesia displayed after intraplantar NGF application is mediated though mast-cell degranulation. This first phase was reduced in intensity by about 66%. There is a possibility that BDNF released at the periphery may be participating in increasing the sensitivity of terminals to noxious stimuli in an NGF-dependant manner or altering the response to non-noxious stimuli (Woolf et al., 1994). These fast effects are mediated by glutamate release in the dorsal horn and retrograde transport and release of BDNF in the dorsal horn from vescicles at the efferent terminals may augment this response in the longer term (minutes).

The second phase of the thermal hyperalgesia elicited upon peripheral NGF application begins around 2hrs p.i and is resolved 24hrs p.i. This phase is mediated though central changes in the dorsal horn. The reduction in this second phase noted when BDNF is removed indicated that BDNF released from the central terminals of $Na_V1.8$ positive neurones in an activity dependant manner is important in the establishment of central sensitization. This effect of BDNF on second-order neurones is mediated though a combination of increases in TrkB expression (Lee et al., 1999) and is accompanied by c-fos expression changes in the dorsal horn and activation of kinase cascades which phosphorylate and activate receptors such as NMDA (Kerr et al., 1999) and concurrently upregulate gene transcription (Yajima et al., 2002). The removal of BDNF expressed in

$Na_V1.8$ positive nociceptors, is sufficient to significantly influence the development of the inflammatory response.

The involvement of BDNF in a wide range of developmental and neuro-modulatory roles, has implicated it in a diverse series of pathways. This widespread expression pattern has made it difficult to tease out the functional effects of BDNF. The development of a mouse which displays the functional deletion of the BDNF gene only in a subset of sensory neurones, the nociceptors, has made it possible to begin to address the question of the role of BDNF in nociceptive processing.

Chapter 6: Conclusions and future work

The genetic manipulations of eukaryotic genomes both *in vitro* and *in vivo* are powerful tools that aid in the elucidation and understanding of nociception and pain pathways. The development of animal models of pain allows direct observation of the behavioural roles of various gene products in mediating nociceptive responses in a variety of contexts. The classification of cell lineages within the nervous system and in particular the peripheral nervous system has demonstrated the heterogeneity of the system and illustrated the need for precise methods of targeting specific neuronal subtypes. The development of more precise analgesic targets identified though genetic manipulation in defined cellular populations, coupled to novel drugs which modulate these interactions, will advance pain management and improve the clinical prognosis of patients especially within intractable, chronic pain conditions.

The search for tools which can target defined tissues or subpopulations of cells *in vivo* is the subject of current research. This work investigated the utility and specificity of three approaches in the study of peripheral nociceptive mechanisms, focussing on modification of DRG sensory neurones in particular. As the site of the initiation of the response to noxious stimuli, first order neurones of the afferent pathway remain an important target for analgesic intervention.

In this thesis three different approaches were used to affect gene expression in the peripheral nervous system. Viral delivery, antisense-mediated intervention and gene ablation using conditional transgenic animals were all assessed for ease and efficiency of use. Additionally, they were employed to achieve modifications in the peripheral nervous system leading to observable behavioural modifications both in physiological and inflammatory states.

6.1 Herpes simplex virus based intervention

The use of viral vectors based on the Herpes simplex virus (HSV) has been promoted in current literature as an efficient, relatively non-invasive means of targeting the neurones of the PNS (Wharton et al., 1995; Coffin et al., 1996; Glorioso and Fink, 2002). The results presented in chapter 2 of this thesis, demonstrated that one of the latest

generation HSV vectors, does not achieve the level of expression necessary to efficiently mediate genetic manipulation in DRG. This vector also failed to target any particular neuronal population preferentially in comparison to any other. These vectors did however transduce neurones preferentially to other non-neuronal cells, albeit at relatively low percentages.

The low level of transduction achieved by these vectors precludes their use to investigate of the roles of nociceptive genes in pain pathways *in vivo*. The non-transfected proportion of cells will continue to produce the protein under investigation leading to ambiguous results. The use of these vectors in studies involved in the over-expression of a gene product especially neurotrophins or other secreted proteins should be more amenable. Indeed there have been several successful protocols both *in vitro* as well as *in vivo* which have exploited the properties inherent in these vectors to achieve a manipulation of secreted molecules and influence nociceptive signal processing (Chattopadhyay et al., 2002). These experimental paradigms were successful because the main objective of the intervention is a gross increase of the particular protein and could be achieved through the use of a high-level expression cassette to drive its production. The actual number of cells transduced is less important (although a reasonable infection percentage must be achieved) than the overall increase in the product in the tissue.

Treatment of chronic pain conditions in animal models, including inflammatory-neuropathic and cancer-related pain, using a replication defective HSV vector expressing human preproenkephalin has proved effective for several weeks following injection (Goss et al., 2001). Similar effects have been demonstrated with vectors expressing neurotrophins (Chattopadhyay et al., 2002; Chattopadhyay et al., 2003; Chattopadhyay et al., 2004). These results have been promising enough to allow phase 1 human trials to be contemplated, again using over-expression of neurotrophic factors (Glorioso et al., 2003a).

The advantages associated with HSV vectors including their ease of introduction, immune response evasion and preference of neurones over other nervous tissue has been identified as the rationale for the continued development of vector systems based on HSV. Results presented in chapter 2 have also demonstrated that neurones are preferentially targeted albeit at low percentages.

In order to use these vectors to ablate nociceptive genes successfully, either with a view to investigating their function or as a clinical therapy to reverse plastic changes associated with non-beneficial pain, the development of safe, highly efficient and targeted vectors becomes imperative. The current vectors examined in this study can be improved by increasing their cell-type specificity, increasing the infection efficiency of the more-disabled versions and defining regulatory sequences which direct expression specifically to defined neuronal populations.

The use of pseudotyping may be one method to achieve the enhanced infection of HSV-based vectors in neuronal cells. Pseudotyped vectors have their genome altered to include surface proteins and proteoglycans from other viruses in an attempt to alter the host cell range. VSV-G pseudotyped Lentiviruses and Adeno-associated viruses have exhibited increased specificity for neuronal infection (Wong et al., 2004; Falk et al., 2002). Investigations along similar lines may lead to the establishment of pseudotyped HSV vectors which exhibit more desirable infection profiles (Anderson et al., 2000).

The efficiency of transfection of HSV derived vectors falls as the level of replication competence falls (Palmer et al., 2000). Unfortunately the expression of HSV genes may be necessary for the efficient transport of the HSV particle from peripheral infection sites to the DRG, or the efficient infection of cells. Any expression of HSV genes is cytotoxic and therefore should be minimised. This requirement becomes an increasingly important consideration if these therapies are to be considered for clinical relevance.

The improvement of infection profiles achieved with multiply-disabled vectors was attempted by fusing the transgene to the VP22 shuttling protein before incorporation in the vectors. This was done to effect a second phase of viral-independent transgene expression in non-infected cells widening the transduced population. The construction of this vector proved problematic probably due to the recombination of the VP22 sequence in the transgenic shuttle vector with endogenous VP22 sequences in the terminally repeated regions.

At present the only method available to increase the infection of DRG neurones with the available HSV-based vectors employs a more invasive surgical route, directly into the DRG body itself (Burton et al., 2002). This technique required the removal of small portions of bone and can lead to a widening of the response curve of the animals to behavioural paradigms. These protocols also result in a widening of the response curve of

the test population necessitating larger numbers for statistical relevance, and are not as easily applicable clinically in humans. The use of more invasive surgical introduction methods also removes a key advantage of HSV vectors over AAV or lentiviral based vectors which show a comparatively better infection rate and toxicity profile but must be delivered by these methods dut to their limited movement away from the primary introduction site (Xu et al., 2003a; Fleming et al., 2001).

The specificity of expression of a transgenic sequence can also be improved by using neuron-specific promoter, enhancer or regulatory regions (Mori et al., 1990). The study of neuron specific genes, such as peripherin or $Na_V1.8$, which target defined subpopulations of neurones (especially nociceptors), may provide the regulatory sequences which can be used to drive expression specifically in these populations of cells.

Investigations into the sequences of these elements and their presence or absence in various nociceptor-specific proteins may illuminate common motifs which may define their function. These may then be used in constructs with a view to limiting transgene expression to cell-types of interest. This approach has been used to investigate the peripherin specification regions upstream of the gene (Lecompte et al., 1999) and has been used to develop Cre-targeting sequences (Zhou et al., 2002).

The current application of HSV-derived vectors in alieviating clinical pain remains limited by several key drawbacks of the system. The fundamental problem of toxicity remains as the most efficient vector subtypes retain a degree of competence. Future research directed at unravelling the basic biology of HSV infection and latency/reactivation cycles should provide important clues which will allow the engineering of a more generic disbled vector backbone which retains the advantageous aspects of HSV biology whilst eliminating detrimental side-effects (Wagstaff et al., 1999; Glorioso et al., 2003b). These vectors when combined with expression cassettes with limited and defined target neuronal subpopulations would advance the clinical relevance of HSV-based genetic modification protocols.

6.2 Transgenic studies

Two transgenic animal lines expressing Cre-recombinase were analysed for their efficiency of expression in neuronal and specifically nociceptive-related tissue. The first line expressed Cre under the control of the Nxc promoter. The Ncx gene is a homeobox domain sequence which is expressed in neural crest derived tissue (Hatano et al., 1997b). These studies were carried out on embryos and neonatal animals, but the expression pattern in adult tissues was not assessed.

Using immunohistological techniques and a reporter strain (ROSA-26R) the Ncx-Cre expression pattern was defined in a variety of adult mouse tissue. Active Cre-enzyme was detected in a wide variety of neural crest– and placode- derived tissue as well as in the brain but not spinal cord. However, the expression in the DRG bodies was not equivalent at all levels assessed; in fact Cre activity was detected only from the lower thoracic (after Th6) until the sacral DRG. The greatest concentration appeared to be centred on the last thoracic DRG. The expression pattern in the gut was robust with most neurones in the myenteric and sub-myenteric plexii exhibiting a positive Cre presence.

Taken together these results may indicate that the positive DRG cell bodies observed bear axons which terminate in the gut and represent a subpopulation of neurones. The efficient transduction of this subpopulation of neurones by this expression cassette makes it potentially useful in the search for methods of investigating and/or treatment of inflammatory disorders of the gut such as colitis, or cystitis.

The second transgenic line investigated expressed Cre-recombinase under the promoter derived from the $Na_v1.8$ gene. This gene is restricted to nociceptors and is present in the majority of this population (Djouhri et al., 2003). This cell line has been characterised extensively and has been demonstrated to exhibit an identical transgenic expression pattern to the endogenous $Na_v1.8$ gene (Stirling et al., 2003).

In order to investigate the contribution of this subpopulation of DRG neurones in the establishment and maintenance of pain, this strain was crossed to a BDNF floxed line to obtain animals which had all BDNF alleles excised in any cell expressing $Na_v1.8$ (the major subset of small diameter nociceptors). BDNF is an important neurotrophin which has been implicated in nociceptive signal processing. Unfortunately the complete understanding of the role of this neurotrophin in the adult nervous system has been

impaired by the non-viability of homozygous BDNF knock-out animals due to the central role played by BDNF in development.

Comparisons between the $Na_v1.8$ BDNF knock-out mice to control littermates demonstrated that BDNF plays a role in some pain pathways. In particular it was determined that removal of BDNF derived from $Na_V1.8$ positive neurons did not lead to any changes in response to a variety of noxious stimuli that result in reflex pathway activation e.g noxious thermal (Hargreaves' test), mechanical (Randall-Sellito) or chemical (first phase of Formalin test). However, the thresholds for responses to various noxious stimuli processing involving supra-spinal circuitry were affected. The hot-plate test (noxious thermal) illustrated a decreased threshold level, resulting in lowered respose times (Hot-plate test). Mechanical stimulation (von Freys) demonstrated that this threshold level was increased at low forces (hair weights under 1 gram) resulting in less sensitive animals. This difference was abolished at noxious mechnical stimuli levels (hair weights over 1 gram). This result implicates BDNF derived from Nav1.8 cells in the transmission of non-noxious mechanical stimuli.

The presence of BDNF derived from $Na_v1.8$ positive cells is important in modulating the pro-inflammatory effects of peripherally applied NGF. Both the first and second phases of thermal hyperalgesia induced with peripherally applied NGF were affected and reduced by the ablation event. This finding implicates C-fibre derived BDNF in inflammatory signal processing. The second phase of the formalin test (noxious chemical stimulation) which involves both ongoing sensory input from the periphery as cell as central sensitization effects was also reduced, futher implicating BDNF.

The use of the $Na_v1.8$-Cre expressing mouse clearly allows the effective manipulation of genes in nociceptors *in vivo* as evidenced by these results. The establishment of the involvement of BDNF released from $Na_V1.8$ positive neurones in inflammatory pathways validates this protein as a potential target for theurapeutic interventions in inflammatory pain.

6.3 Antisense approaches to nociceptor manipulation

The use of antisense oligonucleotides to effect protein knock-down was been widely reported in the literature. The delivery of antisense molecules to nociceptors was

attempted to determine the ease and utility of such an approach in examining pain pathways. The interaction between p11 and $Na_V1.8$, recently shown to be important in mediating TTX-resistant currents important in inflammatory pain (Okuse et al., 2002), was chosen at a site for antisense mediated intervention *in vivo*. Previous work had established this pathway as important in inflammatory pain (Okuse et al., 1997; Akopian et al., 1999). Investigations were done in an attempt to achieve a high level of nociceptor transduction with an antisense molecule directed at p11 and *in vivo* behavioural modifications to inflammatory models were investigated to define the role of the p11 in this pathway

The use of antisense oligonucleotides to achieve efficient and effective ablation of various gene products has many advantages over current transgenic technologies. These are centred upon the ability to use this technique in a wide range of animals including humans compared to transgenics which are currently available only in mice. Reversibility of the antisense effect, absence of developmental effects, the quick and cheap testing of putative sequences and potential to target multiple genes easily in comparison with the slow and expensive transgenic technology.

The main disadvantages associated with this technique centre around the partial knock-down generally achieved with this technique and the inability to target the oligonucleotides to specific subsets of neurones, their inability to achieve over-expression of a gene and the general toxicity of the antisense molecules themselves.

In this study, the manipulation of the $Na_v1.8$ protein levels in subcellular regions of DRG cells was demonstrated though the use of Western analysis. This effect was achieved though the knock-down of intracellular p11 levels by antisense molecules directed against the N-terminal sequence of the mRNA. Efficient delivery of the antisense molecules to DRG was achieved though the use of intrathecal transfusion techniques.

Of the two sequences chosen, only the one encompassing the start codon had any effect. Additionally, control sequences did not have any effect demonstrating the specificity of the mechanism and allowed for changes due to toxicity of the oligonucleotides to be controlled for.

Behavioural consequences accompanied the knock-down of $Na_v1.8$ protein in DRG cells. The normal nociceptive levels in unchallenged animals remained the same before and after treatment, but the response to inflammatory signals was reduced. This implicated

$Na_v1.8$ (as well as other p11-interacting proteins) in this process. The upregulation of $Na_v1.8$ observed on the treatment with NGF may underlie the development of thermal hyperalgesia in inflammatory conditions. The efficient insertion of these channels is dependant on the presence of p11 as shown by the reduction in the channel levels in the membrane fraction of antisense-treated animals, in spite of a general increase in protein levels in whole cell extracts.

Future work to clarify the changes in the expression pattern of other proteins which have been shown to interact with p11 such as TASK-1 potassium channels (Girard et al., 2002), annexin II (Bianchi et al., 1995) or calcium channels (Van den Graaf et al., 2003) may indicate that they are also affected by p11 down-regulation in DRG and the effect observed is due to a more general mechanism involving cellular transport mechanisms.

6.4 Conclusion

This thesis has investigated three approaches to effect genetic modulation in DRG and study the nociceptive-specific functions of various genes. The results obtained have demonstrated that the transgenic approach is the most controllable and hence most informative technique. The continued development of limited transgenic strains will aid in the elucidation of pain pathways.

In contrast, the use of antisense molecules remains quick and efficient and can be used to rapidly screen sequences for *in vivo* roles for putative nociceptive genes cheaply, but continues to suffer from important drawbacks. The data collected with such approaches also remains partially inconclusive due to the inability to adequately limit the effects of the molecules onn non-target populations.

The use of HSV-based viral vectors should provide a link between these two approaches. Viral vector systems will allow the more precise manipulation of genomes, hence their continued development for gene therapy. However, at present the latest generation of HSV-derived vectors do not allow the efficient manipulation of nociceptive genes in an informative manner. The many advantages inherent in the system may make further studies to resolve the current drawbacks worthwhile.

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